Proceedings
of the
Society
for
Experimental Biology and Medicine

VOL. 96 NOVEMBER 1957 No. 2

SECTION MEETINGS

DISTRICT OF COLUMBIA	
George Washington University	October 3, 1957
PACIFIC COAST	
Stanford University	August 29, 1957
SOUTHWESTERN	
Texas A. & M. College	November 1, 1957
WESTERN NEW YORK	
Syracuse University	May 4, 1957

Isolation of Properdin From Human Plasma.*† (23453)

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(Introduced by J. R. Pappenheimer)
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The presence in human serum of a protein, properdin, which together with complement or substances resembling complement, in the presence of Mg^{++} constitutes a natural defense mechanism of blood, was first reported by Pillemer and coworkers(1). A procedure was described for the separation and concentration of properdin activity from human serum. The procedure involves adsorption of properdin on the insoluble yeast polysaccharide, zymosan, in the presence of other serum factors and Mg^{++}(2).‡

The magnesium ion concentration essential for adsorption of properdin is absent from collected human plasma, either because magnesium is bound by a chelating agent such as sodium citrate or because the cations have been removed by adsorption on a cationic exchange resin. For this reason, Pillemer and his colleagues studied the isolation of properdin only from human serum where the cationic constituents of blood are essentially unchanged. To make properdin available in the quantities essential for its careful clinical and chemical characterization, a scheme for its preparation from plasma without alteration of other plasma components is desirable. Whereas schemes of plasma fractionation permitting the isolation of many components from a single plasma pool have been in common use for the preparation of plasma fractions on a

* This work has been supported mainly by Research Contract with Office of Surgeon General, Department of the Army, by contracts with the Office of Naval Research and grants from Commission on Immunology, AFEB, from the Nat. Heart Inst., P.H.S., from Kresge Fn., from the King Fn. and by contributions from industry.

† The authors are deeply indebted to the late Dr. Louis Pillemer of Western Reserve University for his interest in this work and in the advice which he has given freely, to Mr. Lewis Larsen of the Biologic Laboratories of the Mass. Dept. of Public Health, and to Mr. Marshall Melin of Protein Foundation Laboratories for their assistance in providing plasma fractions.

‡ Pillemer, L., and Blum, L., to be published.

large scale since World War II, serum has not been similarly fractionated since all the components of the clotting system have been removed or altered in its preparation. Plasma is thus available in quantity in many laboratories while serum is not.

The present paper reports the successful application of the procedure described for serum to plasma from blood collected through a cationic exchange resin and to plasma from blood collected in ACD solution. Certain modifications have been introduced which increase the efficiency of the technic in terms of time and volume. Preliminary studies which indicate the successful separation of properdin activity from plasma without the use of zymosan are reported.

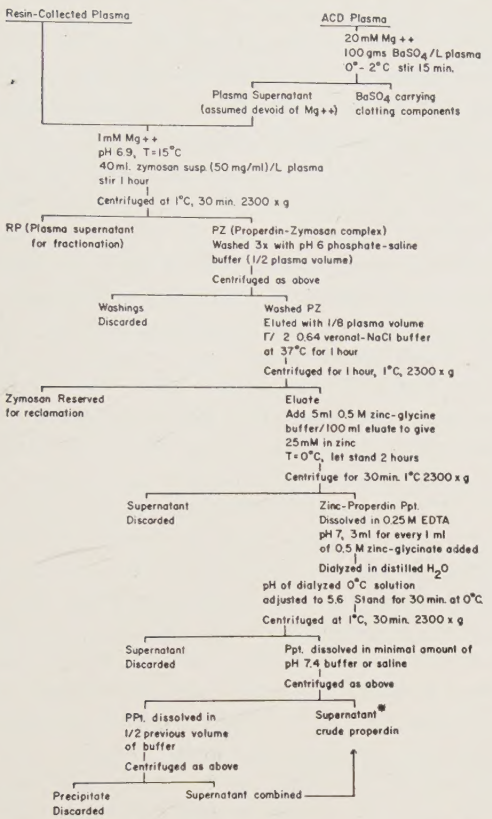
Materials. The blood employed was collected either in ACD solution, (acid-citrate-dextrose solution, prepared according to NIH formula A) or through cationic exchange resins (IRC-50 or Dowex-50) in the ADL-Cohn Blood Fractionator(3). Zymosan was obtained commercially.§ Water baths capable of maintaining the desired temperature to ± 1°C were used for temperature control during both adsorption and elution of properdin. Zinc glycinate(4) reagent was used for the introduction of zinc ions. The stock barbital buffer for suspension of zymosan has been used as described(5), with the exception that magnesium and calcium are omitted. The barbital buffer used during elution is as described(5). *Procedures.* The zymosan adsorption technic for the preparation of properdin described by Pillemer *et al.*(5) was followed closely with only those modifications specifically noted. The procedure has been found to be perfectly reproducible. Application of the procedure to human plasma involves the maintenance of a sufficient concentration of Mg^{++} ions without the induction of clotting. *A. Application to Resin-Collected Plasma.* Under the standard conditions of flow rate and contact time used in collecting blood by passage over a sodium cycle cationic exchange resin, the concentration of Ca^{++} + Mg^{++} ions is reduced to approximately 5 x 10^{-5} M(6). Addition of Mg^{++} ions to resin-collected plasma to a concentration of 10^{-3} M does not initiate any overt coagulation process. The zymosan adsorption of properdin may then follow with successful removal of properdin activity from the plasma. In 20 runs, in which the resin-collected plasma had an average properdin activity of 4 units per ml,‖ less than 1 unit per ml of properdin remained in the plasma after zymosan adsorption. *B. Application to ACD Plasma.* In the presence of sodium citrate, magnesium and other cations are bound by the citrate ions. Saturation of the citrate ions with added Mg^{++} ions leads to liberation of Ca^{++} ions, the association constant of the Ca-citrate complex being smaller than that of the Mg-citrate complex. The liberated Ca^{++} ions participate in the initiation of the coagulation process. This may be prevented by prompt adsorption of prothrombin and other clotting components onto $BaSO_4$¶(7,8). As with resin-collected plasma, zymosan adsorption of properdin may then be successfully applied. Details of the technic are given in the flow sheet (Table I). It is to be noted that the $BaSO_4$ adsorbed plasma is considered to be devoid of free Mg^{++} ions and is, therefore, made 10^{-3} M in Mg^{++} ions before incubation with zymosan. In 4 runs, in which the ACD-collected plasma had an average properdin activity of 4 units per ml,‖ less than 1 unit per ml of properdin remained in the plasma after zymosan adsorption. C. Table I presents a flow sheet of the technic employed for isolation of properdin from both types of plasma in our laboratory. The essence of the method remains the same as originally presented by Pillemer *et al.* The few modifications are noted. (1) *Temperature of incubation for adsorption of properdin on Zymosan*: The temperature at which properdin has been adsorbed onto zymosan

§ Zymosan from lots 5B171 and 6B14 has been purchased from Fleischman Yeast Division, Standard Brands, Inc.

‖ It has been found difficult for technical reasons to obtain accurate properdin determinations in plasma, particularly in ACD plasma. The properdin values given for plasma before zymosan adsorption should be considered to represent minimal values.

¶ Baker, C. P. $BaSO_4$ has been found superior to other brands for this purpose.

TABLE I



*Further concentration of properdin activity can be achieved by high speed centrifugation in the Spinco preparative ultracentrifuge. (6)

is 15°C. (2) *Adjustment of pH*: Acetate buffer (pH 4, 0.2 ionic strength) has been substituted for 1.0 N hydrochloric acid for adjustment of the plasma to pH 6.9 prior to addition of zymosan. Local concentration of hydrogen ions which might produce irreversible alteration of plasma proteins is less likely to ensue from the use of acetic acid rather than from completely ionized hydrochloric acid. The mixture may, alternatively, be adjusted to pH 6.9 by bubbling CO_2 through it. This procedure also avoids the use of strong acids but has the disadvantages of being time consuming and of possible surface denaturation of protein by the stream of bubbles. As indicated by Pillemer *et al.*, the pH of the zymosan-plasma mixture rises during the hour's incubation at 15°C(1,5). We have found this rise in pH to be due to loss of carbon dioxide during stirring. Maintenance of constant pH 6.9 has been obtained by imposing a layer of carbon dioxide over the mixture while the pH is monitored by an automatic titrator.** (3) *Elution volume*: It has been found that a volume of buffer equal to 1/8th the volume of original plasma is adequate for elution of properdin from the zymosan-properdin complex.†† The buffer is made by mixing 0.09 plasma volume of veronal buffer (pH 7.4, 0.19 ionic strength) with 0.035 plasma volume of 2 M NaCl solution. The buffer is warmed to 37°C before suspension of the washed zymosan-properdin complex(5). (4) *Precipitation of properdin by zinc ions*: Properdin has been found to form a water-insoluble zinc complex. Thus when zinc glycinate reagent was added directly to the eluate to a final zinc concentration of 25 mM at pH 7.1-7.5, quantitative precipitation of zinc-properdin occurred. The precipitated properdin complex may be dissolved in small volume in the presence of ethylene-diamine-tetra-acetic acid (EDTA), thus greatly reducing the volume of fluid which must be dialyzed with a concomitant reduction in time of dialysis. The volumes suggested in the flow sheet result in solution of the properdin-zinc complex at 1/50 plasma volume. In large scale fractionation, where plasma volumes may be as great as 300 to 1000 liters of plasma, this solution may be accomplished satisfactorily at 1/500 plasma volume. (5) *Solubility of properdin in water*: After dialysis is completed, the dialyzed material is adjusted to pH 5.6 by careful addition of acetate buffer. Elevation of the ionic strength of the mixture must be avoided insofar as possible. Qualitative observations indicate that, in water, the pH of minimum solubility of properdin is close to 5.6(9). This is highly suggestive that the iso-electric point of properdin is near this pH value. Addition of a small amount of salt to the precipitated complex will dissolve the properdin at pH 5.6, showing the protein to exhibit the classical euglobulin behavior.

Results. Comparison of resin-collected and ACD plasma as sources of properdin. In

** Radiometer Titrator manufactured by Radiometer Co., Copenhagen, Denmark.

†† E. W. Todd and L. Pillemer, to be published.

TABLE II. Comparative Properdin Recovery from ACD and Resin Plasma Prior to Ultracentrifugation.

Age of plasma	Units of properdin recovered/ml of original plasma: ACD plasma	Resin plasma
1 to 2 days	2.18 (2 runs)	2.45 (11 runs)
1 to 2 wk	1.05 (2 ")	1.63 (7 ")
1 mo or more		.71 (3 ")

Table II, it is evident that properdin may be prepared from both fresh ACD and fresh resin-collected plasma although recoveries were somewhat better when the source material was resin-collected. Establishment of the feasibility of ACD plasma as a source of properdin is important, however, for this is the form in which essentially all of the human plasma available for fractionation occurs. It has been found difficult for technical reasons to obtain accurate properdin determinations in plasma, particularly in ACD plasma. The values obtained are often lower than the actual titer as witnessed by the eventual recovery of more than the original amount of properdin as calculated from the plasma value. To avoid confusion from this source, recovery of properdin activity is expressed (in Table II and other tables) as units recovered per milliliter of starting plasma, assuming that original pooled plasma properdin titers will vary only within a limited degree.

Age of plasma as a factor in properdin yields. Although human plasma should be fractionated promptly upon collection for optimal results, a major source of plasma for fractionation is from outdated blood (3 weeks or more after collection) from hospital blood banks. It has been shown that albumin and gamma globulin can be satisfactorily prepared from such plasma(10,11). Table II presents the recovery of properdin from plasma of varying age. The decrease of yield with the age of the plasma is undoubtedly due, at least in part, to the loss of plasma factors essential for properdin adsorption on zymosan. Some of the components of complement are known to become inactive during plasma storage. Nevertheless sufficient properdin can be recovered from outdated plasma to suggest that this major plasma source can be of value for large scale preparation of properdin.

Comparison of properdin recovery following zinc precipitation and following dialysis of the total eluate. From Table III, it is evident that essentially identical recovery of properdin was obtained whether or not the original eluate was dialyzed *in toto* or whether advantage was taken of the zinc precipitability of properdin to reduce the volume to be dialyzed.

Recovery of properdin in fraction I. Cold ethanol Method 6 of Cohn and collaborators (12) has been used in a preliminary study of the feasibility of preparing properdin by direct plasma fractionation without recourse to the use of zymosan. The major portion of the properdin activity is to be found in Cohn Fraction I when this fraction is precipitated at pH 6.9 or lower. This has proved true in 7 consecutive runs both for fresh resin-collected plasma and for plasma from outdated ACD blood. In 2 experiments designed to isolate properdin activity, 90-95% of the total properdin activity of the original plasma was recovered in a subfraction of Fraction I. Eventual recovery of properdin by direct fractionation appears extremely hopeful, thus eliminating the need for the costly and variable zymosan, except for assay purposes. Recovery of properdin from outdated plasma by direct fractionation will presumably not be dependent on the labile plasma components essential for the interaction of properdin with zymosan.

Pillemer has reported(1) the major properdin activity to be localized in Fraction III prepared from serum by the method of Deutsch(13). In our laboratory, as well, the

TABLE III. Comparison of Properdin Yields by Dialysis Procedure and by Zinc Precipitation Procedure Prior to Ultracentrifugation.

Run	Units of properdin recovered per ml of original plasma: Dialysis procedure	Zinc precipitation procedure
323 P	3.0	2.1
327	2.5	1.1
328	2.1	2.5
329	3.7	4.7
330	2.5	2.6
HP-2	.5	.6
331	2.5	3.5
Avg	2.4	2.4

properdin activity not found in Fraction I appears in Fraction III. Careful definition of the variables responsible for the distribution of properdin during fractionation of plasma is being pursued.

Summary. Methods have been presented for the preparation of properdin from resin-collected and from ACD plasma by the application of the zymosan adsorption technic of Pillemer *et al.* Preliminary data are presented suggesting the feasibility of the isolation of properdin from Cohn Fraction I without the use of zymosan.

1. Pillemer, L., Blum, L., Lepow, I. H., Ross, O. A., Todd, E. W., and Wardlow, A. C., *Science,* 1954, v120, 279.

2. Pillemer, L., Lepow, I. H., and Blum, L., *J. Immunol.,* 1953, v71, 399.

3. Tullis, J. L., Surgenor, D. M., Tinch, R. J., D'Hont, M., Gilchrist, F. L., Driscoll, S., and Batchelor, W. H., *Science,* 1956, v124, 792.

4. Antoniades, H. N., Pennell, R. B., McArthur, J. W., Ingersoll, F. M., Ulfelder, H., and Oncley, J. L., *J. Biol. Chem.,* in press.

5. Pillemer, L., Blum, L., Lepow, I. H., Wurz, L., and Todd, E. W., *J. Exp. Med.,* 1956, v103, 1.

6. Buckley, E. S., Jr., and Gibson, J. G., II, *Conference on New Mechanized Equipment for Collecting and Processing of Human Blood.* Univ. Lab. of Phys. Chem., Harvard University, 1951, p25.

7. Surgenor, D. M., and Noertker, J. F., *J. Am. Chem. Soc.,* 1952, v74, 3448.

8. Alexander, B., Goldstein, A., and Schmid, K., *J. Phys. Colloid Chem.,* 1951, v55, 94.

9. Pillemer, L., *Ann. N. Y. Acad. Sci.,* 1956, v66, 233.

10. Mulford, D. J., and Dwyer, R. C., *Am. J. Pub. Health,* 1950, v40, 691.

11. Banton, H. J., and Mulford, D. J., *ibid.,* 1950, v40, 697.

12. Cohn, E. J., Strong, L. E., Hughes, W. L., Jr., Mulford, D. J., Ashworth, J. N., Melin, M., and Taylor, H. L., *J. Am. Chem. Soc.,* 1946, v68, 459.

13. Deutsch, H. F., Gostings, L. J., Alberty, R. C., and Williams, J. W., *J. Biol. Chem.,* 1946, v164, 109.

Received July 15, 1957. P.S.E.B.M., 1957, v96.

Some Factors that Influence Utilization of Antihemophilic Activity During Clotting.* (23454)

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When normal blood or plasma clots, it loses its antihemophilic activity(1). If plasma is first adsorbed with $BaSO_4$, antihemophilic factor (AHF) remains in the adsorbed plasma even after recalcification; it thus is stabilized by the adsorption procedure. This paper describes the effect on AHF utilization of adding various plasma and serum preparations and derivatives to this stabilized plasma. It was found that only when a source of thrombin was added would AHF disappear. Within the lower range of effective thrombin concentrations, the rate of loss of AHF was proportional to the amount of thrombin available. Preliminary reports of some of these data have been made previously(2).

Materials and methods. Oxalated plasma and *native, platelet-poor plasma* were prepared as previously described(1). *PTC-deficient plasma* was obtained from a patient with a moderately severe disease characterized by hemarthrosis and hematuria. *Plasma deficient in Stuart factor* was from the patient described by Hougie and associates(3). *"Dicumarol plasma"* was prepared from a dog to whom 1400 mg dicumarol had been administered over a 13-day period. *"Stabilized plasma"* was made by twice adsorbing oxalated plasma with $BaSO_4$ (Merck R; 100 mg/ml at 28°C for 20 min). *Normal and hemophilic canine sera* were obtained from blood allowed to stand in glass for 24 hours. *Serum accelerator factor* (*SAF*) was prepared as described earlier(4). *Plasma thromboplas-*

* This investigation was supported by the Research and Development Division, the Office of the Surgeon General, Department of the Army.

† Markle Scholar in Medical Science.

TABLE I. Effect of Various Plasmas, Sera, or Fractions on AHF in Stabilized (Adsorbed) Plasma.

Exp. No.	Material tested	% prothrombin in test material	Residual AHF as % initial level		
			Reaction period in min.		
			5	15	30
I. Controls					
1	Oxalated saline (mean of 9 tests)	0	—	—	98 ± 11.7
2	Normal canine plasma (" " 4 ")	100	11 ± 6.2	8 ± 4.8	<5
3	" human " (" " 3 ")	100	<5	8 ± 4.6	<5
II. Normal serum and serum fractions					
4	Normal canine serum	<5	95	82	70
5	Canine SAF (100 Alexander U/ml)	<5	84	89	97
6	Adsorbed canine serum	<5	—	97	93
7	Human PTC preparation	<5	88	82	82
III. Deficient plasmas and sera					
8	Dicumarol canine plasma	<5	98	97	97
9	Hemophilic " "	105	<5	<5	<5
10	PTC-deficient human plasma	115	<5	<5	<5
11	Hemophilic canine serum	18	17	<5	<5
12	Stuart-factor deficient human plasma	86	39	22	12

tin component (*PTC*) was made by the method of White, Aggeler, and Glendening (5). The *prothrombin* used was adsorbed from canine oxalated plasma with $Al(OH)_3$ and eluted with phosphate buffer; it contained 3000 Iowa units/mg N. *Thrombin* was obtained by activating a canine prothrombin preparation with 25% sodium citrate(6); it contained 5000 units/mg N. *Bovine AHF* was made by a modification of Bidwell's procedure(7) and was diluted to approximate the activity of canine plasma. *Oxalated saline* consisted of 1 part 0.1 M sodium oxalate in 99 parts saline (0.15 M NaCl). *Crude cephalin* was made from dog brain(8). The two-stage method of prothrombin determination(9) was used. Assays of AHF were performed by the method of Langdell, Wagner and Brinkhous(8).

Procedure. The following *test system* for studying AHF utilization was employed. All plasma and test materials were first dialyzed against oxalated saline for 2 hours at 4°C with constant agitation. Reagents were mixed in the following order and amounts: 1.4 ml stabilized plasma, 0.1 ml imidazole buffer (pH 7.3), 0.1 ml 0.3% cephalin, 0.1 ml test material, 0.24 ml 1.2% $CaCl_2$. After varying reaction periods up to 30 minutes, 0.4 ml 0.1 M sodium oxalate were added and residual AHF assayed after adsorption with $BaSO_4$. Antihemophilic activity at zero time represented 100% AHF. Slight variations in this basic procedure are indicated in the text. Whenever reagents were omitted, oxalated saline was substituted to maintain constant volume.

Results. The effect on AHF utilization of adding various plasmas and sera to the test system is shown in Table I. Illustrative experiments only are given. Exp 1 demonstrates that AHF is stable in adsorbed plasma, even in the presence of cephalin and calcium. In another experiment of this type, not included in the table, 109% AHF was found after a reaction period of 6 hours. The rapid disappearance of AHF caused by only a small amount of canine or human plasma is illustrated by Exp 2 and 3. When normal canine serum or its fractions were tested (Exp 4, 5, and 6), little or no loss occurred. The same relative stability was encountered when a human PTC preparation (Exp 7) or dicumarol plasma (Exp 8) was added. None of the materials tested in Exp 4-8 contained sufficient prothrombin to be detected in the 2-stage assay. Hemophilic plasma (Exp 9) and plasma which was moderately deficient in PTC (Exp 10) were as effective as normal plasma in causing loss of antihemophilic activity; they contained normal amounts of prothrombin. In contrast to normal serum (Exp

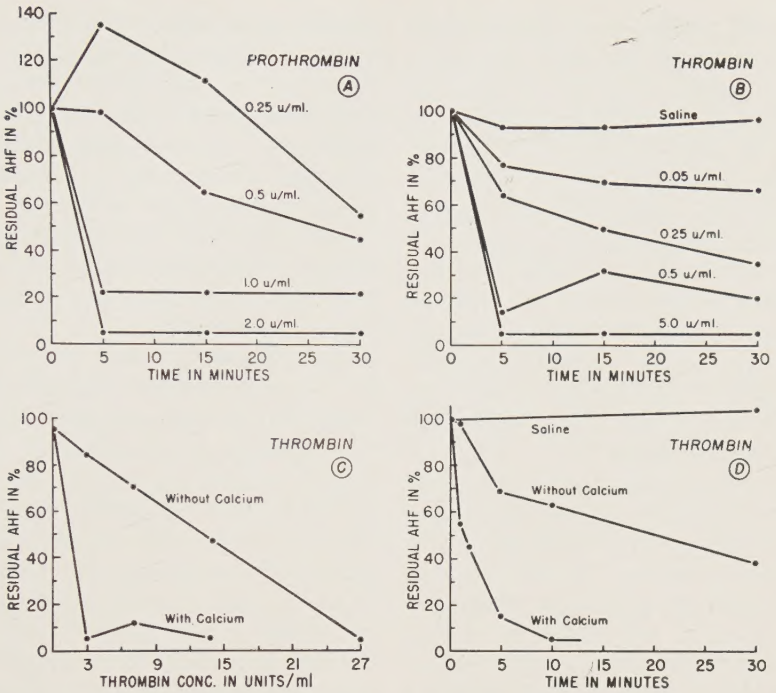

FIG. 1. Effects of purified prothrombin and thrombin on AHF in stabilized plasma. Procedures performed at 28°C in silicone-treated tubes. Indicated concentrations refer to final reaction mixtures. See text for complete test system. A. Effect of various concentrations of purified prothrombin (indicated for each curve) on AHF utilization in the presence of calcium and cephalin. B. Effect of various concentrations of thrombin (indicated for each curve) on AHF utilization with calcium but not cephalin present. C. Comparison of effects of various concentrations of thrombin on AHF utilization with and without added calcium. Reaction time = 30 min. Saline control (no thrombin) contained 95% AHF after 30 min. D. Comparison of AHF utilization rates in the presence of a constant concentration of thrombin (5 u/ml) with and without added calcium.

4), hemophilic serum (Exp 11) produced prompt AHF loss; it contained 18% residual prothrombin. Thus, only those reagents that contained prothrombin effected a loss of antihemophilic activity under these conditions.

Even when prothrombin was present, unless it was readily converted to thrombin, the AHF persisted at high levels. Human plasma deficient in the Stuart factor formed thrombin slowly; although it contained prothrombin, only 54% was converted in 1 hour(3). There was a correspondingly slow drop in the AHF (Exp 12). To show further that AHF loss is related to conversion of some prothrombin to thrombin, both AHF and prothrombin were repeatedly determined in slowly clotting, platelet-poor plasma at 4°C. Under these conditions, both factors were relatively stable. In one experiment, after 2 hours, 103% prothrombin and 120% AHF were present. A flimsy fibrin clot then formed. During the next 30 minutes only 23% of the prothrombin was utilized, but all of the AHF disappeared.

The effects of purified prothrombin and thrombin on the rate of AHF loss were studied next (Fig. 1). Fig. 1A demonstrates the results of adding various concentrations of purified prothrombin to the test system. Increasing the amount of prothrombin caused increasingly rapid loss of AHF. As little as 2 units/ml caused all of the AHF to be lost in 5 minutes. Cephalin and calcium were necessary constituents of these reaction mixtures; without them, the prothrombin was not converted and no loss of AHF occurred. In the experiments shown in Fig. 1B and 1C, the loss of AHF from stabilized plasma was proportional to the thrombin concentration. Cephalin was found to be unnecessary when this preformed thrombin was used, and was

omitted from these mixtures. Without ionic calcium, more thrombin was needed to cause a given loss of AHF (Fig. 1C). Even so, there was a linear relationship between the concentration of thrombin and the AHF lost. When calcium was added to the thrombin-plasma mixtures, all of the AHF disappeared with a thrombin concentration as low as 3 units/ml. Relative rates of AHF loss with a constant amount of thrombin in the presence and absence of ionic calcium are compared in Fig. 1D. Progressive loss of antihemophilic activity occurred in the absence of calcium, but the reaction was markedly accelerated when calcium was available.

Fibrinogen was present in the reaction mixtures of each of the above experiments. Other experiments were done in which fibrinogen was reduced to trace amounts. In one, purified bovine AHF was substituted for stabilized plasma and thrombin was added as in Fig. 1D. The rates of disappearance of antihemophilic activity were similar to those obtained with stabilized plasma. Without added calcium, 56% AHF was lost in 30 minutes; with calcium present, all activity disappeared in less than 10 minutes.

Discussion. These data suggest that thrombin plays a major role in utilization of AHF during clotting. That some factor which is adsorbable onto $BaSO_4$ is required is demonstrated by the stability of AHF in adsorbed plasma after recalcification. This factor appears to be diminished in dicumarol plasma, normal serum or preparations of SAF or PTC, all of which are poor thrombin sources. Plasmas or sera that contain prothrombin, however, whether normal, hemophilic or deficient in PTC or Stuart factor, will restore conditions that permit the AHF to disappear when cephalin and calcium ions are available to cause its conversion to thrombin. Preformed thrombin is able to cause the loss of antihemophilic activity from stabilized plasma or from purified AHF. Perhaps AHF is another molecule subject to the enzymatic splitting action of thrombin. Only small amounts of thrombin are required to inactivate AHF if calcium ions are available, a finding in keeping with our earlier impression that prompt and thorough decalcification is necessary for optimal preservation of AHF in stored blood (10). Commercial preparations of thrombin have been reported to be contaminated with profibrinolysin(11). Fibrinolysin can destroy AHF(12). It would seem unlikely that the thrombin preparation used in these experiments (prepared by adsorption, elution and citrate-activation) had significant fibrinolytic activity. However, effects of possible contaminants of the purified reagents cannot be completely excluded.

Reduction in the levels of circulating AHF has been observed in dogs subjected to cold-injury(13) and thromboplastin injection(15), two conditions that appear to be associated with systemic coagulation. The *in vivo* response to AHF appears to be a sensitive index of intravascular clotting, probably because only a small amount of thrombin is required to cause loss of antihemophilic activity. These conclusions are further supported by the depressions in circulating AHF that have been produced by injecting thrombin preparations intravenously(14,15).

There is some discrepancy between our observations and those made by Douglas(16). He observed a normal rate of disappearance of AHF in the absence of platelets; our experiments indicate a retarded rate if the platelets are sufficiently reduced to delay thrombin formation. This contrast might be explained by differences in methods. A similar explanation probably applies to variations between our data and those of Johnson and Seegers(17). These authors did observe loss of activity of "platelet cofactor I" in the presence of thrombin and calcium, but none if calcium were omitted from the reactions.

It is perhaps possible that AHF passes through a more active phase under the influence of thrombin. Such a concept would be in keeping with an autocatalytic role of thrombin and the formation of a "prothrombin conversion factor" as recently described(18). In Fig. 1A, it will be noticed that the most dilute thrombin elicited a brief hyperactive phase. In other experiments, this hyperactivity was of even greater magnitude, but was evanescent and difficult to reproduce consistently.

Summary. A study has been made of the effects of various plasmas, sera and fractions

on AHF in stabilized ($BaSO_4$-adsorbed) plasma. Only reagents that provided a source of thrombin caused AHF to disappear after recalcification. Materials tested that produced loss of AHF were: normal, hemophilic, PTC-deficient and Stuart factor deficient plasmas and hemophilic serum. All contained prothrombin. Those that produced no significant loss were: dicumarol plasma, normal serum, SAF, PTC and adsorbed serum; they contained no detectable prothrombin. Slow prothrombin conversion (*e.g.* Stuart plasma, platelet-poor plasma) was accompanied by slow AHF utilization. Removal of fibrinogen from reaction mixtures did not abolish AHF loss. Purified prothrombin caused utilization of AHF in proportion to its concentration if cephalin and calcium ions were present to cause conversion to thrombin. Preformed thrombin was also effective in proportion to its concentration; its effect was accelerated by calcium ions. The significance of these findings is discussed in relation to blood preservation, intravascular coagulation and basic clotting reactions.

Grateful acknowledgement is made to Robert H. Wagner for supplying the purified prothrombin, thrombin and bovine AHF and to Cecil Hougie for the purified PTC used in this study, and to Elizabeth H. Hendrick and Ira D. Godwin for their technical assistance.

1. Graham, J. B., Penick, G. D., and Brinkhous, K. M., *Am. J. Physiol.,* 1951, v164, 710.

2. Penick, G. D., *Proc. XXth Internat. Physiol. Congress,* 1956, 716; *Fed. Proc.,* 1957, v16, 369.

3. Hougie, C., Barrow, E. M., and Graham, J. B., *J. Clin. Invest.,* 1957, v36, 485.

4. Graham, J. B., Langdell, R. D., Morrison, F. C., Jr., Brinkhous, K. M., PROC. SOC. EXP. BIOL. AND MED., 1954, v87, 45.

5. White, S. G., Aggeler, P. M., and Glendening, M. B., *Blood,* 1953, v8, 101.

6. Seegers, W. H., McClaughry, R. I., and Fahey, J. L., *ibid.,* 1950, v5, 421.

7. Bidwell, E., *Brit. J. Haemat.,* 1955, v1, 35.

8. Langdell, R. D., Wagner, R. H., and Brinkhous, K. M., *J. Lab. Clin. Med.,* 1953, v41, 637.

9. Wagner, R. H., Graham, J. B., Penick, G. D., and Brinkhous, K. M., *The Coagulation of Blood,* ed. L. M. Tocantins, Grune and Stratton, N. Y., 1955, 105.

10. Penick, G. D., and Brinkhous, K. M., *Am. J. Med. Sci.,* 1956, v232, 434.

11. Siegel, M., and Cliffton, E. E., *J. Gen. Physiol.,* 1957, v40, 377.

12. Wagner, R. H., Pate, D., and Brinkhous, K. M., *Fed. Proc.,* 1954, v13, 445.

13. Brinkhous, K. M., and Penick, G. D., *Thrombosis and Embolism,* Benno Schwabe and Co., Basel, 1955, 428.

14. Alexander, B., Goldstein, R., Rich, L., Le Bolloc'h, A. G., Diamond, L. K., and Borges, W., *Blood,* 1954, v9, 843.

15. Penick, G. D., Wagner, R. H., Roberts, H. P., and Brinkhous, K. M., *Fed. Proc.,* 1955, v14, 415.

16. Douglas, A. S., *Blood,* 1956, v11, 423.

17. Johnson, S. A., and Seegers, W. H., *Rev. hèmat.,* 1954, v9, 529.

18. Therriault, D. G., Gray, J. L., and Jensen, H., PROC. SOC. EXP. BIOL. AND MED., 1957, v95, 207.

Received July 22, 1957. P.S.E.B.M., 1957, v96.

Serial Propagation of the Guinea Pig Salivary Gland Virus in Tissue Culture. (23455)

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The use of tissue culture methods for isolation and propagation of the viruses of mouse(1) and human(2-4) salivary gland disease and the attendant development of technics for their serological study suggested similar efforts to establish the salivary gland virus of guinea pigs. Andrewes(5) found that the inoculation of suspensions of infected salivary glands into modified Maitland cultures of guinea pig testis regularly resulted in development in 3 to 6 days of intranuclear inclusion bodies in mononuclear cells in the in-

terstitial tissues. Active virus was recoverable for at least 5 days by subcutaneous inoculation of young guinea pigs with culture fluids. However, all attempts to pass the virus serially in tissue cultures failed. The present report is concerned with the successful propagation of a strain of the guinea pig salivary gland virus in tube cultures of guinea pig tissue, and the application of serological technics to its identification.

Methods. Tissue cultures. Explant cultures of guinea pig submaxillary gland and embryonic guinea pig muscle were prepared by the plasma clot technic and were grown and maintained in a modification of Enders' beef amniotic fluid medium(6,7). Trypsin-dispersed stationary tube and 8 oz. bottle cultures were prepared from embryonic guinea pig muscle and were grown in 15% guinea pig serum in Eagle's basal medium(8), to which soybean trypsin inhibitor (0.005%) was added; after satisfactory outgrowth of cells, usually in 4 to 7 days, cultures were washed 3 times in Eagle's basal medium and thereafter maintained in 5% horse serum in Eagle's basal medium. The cell sheet consisted almost exclusively of elongated polygonal or spindle-shaped cells, which appeared to be fibroblasts; occasional small islands of epithelium were present in a few cultures. All media contained penicillin (250 units per ml) and streptomycin (250 μg/ml). Tube cultures contained 1 ml of maintenance fluid and were changed twice weekly; bottle cultures contained 20 ml and were changed once weekly. *Virus. Strain 1.* Healthy guinea pigs harboring the salivary gland virus were supplied by a dealer in Yonkers, N.Y. The virus was established in serial passage in guinea pigs from the N.I.H. colony ("N.I.H." strain) by subcutaneous injection into the region of the salivary glands using 0.2 ml of a 10% suspension of submaxillary glands from 2 infected animals; passages were made at 3- to 4-week intervals. Histological examination of submaxillary glands harvested at each passage revealed typical intranuclear inclusion bodies within enlarged duct cells, with varying degrees of mononuclear cell infiltration. No overt disease was associated with infection by this route. The virus carried in serial passage may represent a mixture of strains since 7.7% (3/39) of control "N.I.H." strain guinea pigs aged 5 months or older were naturally infected, as evidenced by infrequent salivary gland inclusions. The tissue culture passage line was initiated by inoculation of guinea pig embryo muscle explant cultures with 0.2 ml of a freshly prepared, lightly centrifuged 10% submaxillary gland suspension from the second guinea pig passage. Serial passages in tissue culture were made with 0.1 or 0.2 ml of culture fluid containing whole or ground cells harvested at the time of severe cytopathogenic effects. *Strain 2.* Infected submaxillary gland material from guinea pigs supplied by another dealer in New York State, was the source of a second serial passage line initiated and carried in "N.I.H." strain animals. *Complement Fixation Test.* The technic was a modification of the Bengtson procedure(9,10). The source of complement was serum from 6- to 8-week-old guinea pigs of a strain ("Hartley") apparently free of infection; the serum was preserved at 4°C in hypertonic salt solution, one part of saturated NaCl to 9 parts serum(9); activity comparable to that of commercial lyophilized complement was maintained for at least 6 weeks. Virus and control antigens consisted of tissue culture cells ground in a TenBroeck grinder and suspended in culture fluid; antigens were usually heated at 56°C for 30 minutes. Standard positive serum for titrating antigens was hyperimmune guinea pig serum or commercial complement having high titer antibody. Serums were inactivated by heating at 56°C for 30 minutes immediately before use. *Neutralization test.* Virus preparations consisted of supernatant fluid of fresh homogenates of severely affected cells in culture fluid, centrifuged at 1000 rpm for 10 min. Tests were performed in trypsin-dispersed cultures, using the technic described for the adenoviruses(11). Because of difficulty in obtaining complete neutralization, the criterion of neutralization was cytopathic effects less than one-half the virus control reading on the day when the latter first showed 2-plus or greater involvement. *Histology.* Tissues were fixed in Zenker's-formalin and stained with Giemsa stain.

Results. Tissue culture isolation of strain 1. Primary inoculation of explant cultures of guinea pig embryo muscle with infected submaxillary gland suspension produced in 10 days small foci of enlarged, round, refractile, smoothly outlined cells in the fibroblast outgrowth. By the 20th day, the focal areas had extended to involve more than one-half the fibroblasts; at 28 days, there was almost complete involvement and many necrotic cells were present. The characteristic changes were reproduced on transfer of fluid containing ground cells to both trypsin-dispersed and explant cultures. Cytopathic changes were not seen in explant cultures until the 24th day after inoculation, but the incubation period in trypsinized cultures was 6 days and all cells were involved in 12 days; consequently, trypsin-dispersed cultures were used for all subsequent passages. After 10 passages, the incubation period shortened to 2 or 3 days, with complete degeneration occurring within 6 to 8 days. The virus was carried through 22 serial passages, representing a theoretical dilution of the original inoculum of 10^{-33}. In stained preparations made at intervals throughout the passage series, the majority of cells contained large, elongated, often kidney-shaped, eosinophilic intranuclear inclusion bodies with marginated chromatin and a clear halo. The inclusions closely resembled those produced by the human salivary gland virus. Definite cytoplasmic inclusions were not observed.

Repeated attempts to infect tissue cultures of human cells with the guinea pig agent, and vice versa, were completely negative.

Complement fixing antigen was demonstrated with regularity in cultures showing severe cytopathic changes; highest antigen titers (1:8 or, rarely, 1:16) were obtained by grinding the cells and suspending in supernatant fluid. Commercial guinea pig complement from each of 5 companies was found to contain antibody against the guinea pig salivary virus antigen prepared in tissue culture, in titers of 1:64 to 1:256. Consequently, serum from young guinea pigs, demonstrated to be free of antibody, was used for complement. The commercial complement served as a positive standard reference serum.

CF antibody-free guinea pigs inoculated with either strain 1 or strain 2 guinea pig passage virus regularly developed CF antibody titers of 1:64 within 3 weeks. Titers of 1:128 or greater were found in guinea pigs hyperimmunized with submaxillary gland suspensions infected with either strain. No reactions were obtained when control guinea pig tissue culture antigens were tested against a number of strongly positive antiserums. No serological relationship could be demonstrated to the human or mouse salivary gland viruses.

Poorly adapted virus in early tissue culture passage was generally unsatisfactory for tissue culture neutralization tests, because of extended incubation periods of cell-free virus suspensions and the difficulty in neutralizing virus in cell suspensions. After the 7th tissue culture passage, there was generally enough virus activity in centrifuged cell homogenates to permit satisfactory tests. Development of neutralizing antibody was demonstrated with regularity in guinea pigs infected with either animal passage or tissue culture passage virus.

Identification of the tissue culture isolate as the virus of guinea pig salivary gland disease was indicated by the characteristic inclusion bodies produced in tissue culture cells and the ability of tissue culture virus to demonstrate neutralizing and complement-fixing antibody responses in animals infected with submaxillary gland virus from two animal passage lines. In order to provide supportive evidence, attempts were made to reproduce with tissue culture passage virus the pathognomonic features of spontaneous salivary gland disease and the acute meningitis resulting from intracerebral inoculation of virus-containing submaxillary gland suspensions (12). Virus from the 10th tissue culture passage was used; this material produced characteristic cytopathic effects in 2 days when inoculated into tissue culture and represented a 10^{-20} theoretical dilution of the original submaxillary gland suspension. Four 4- to 5-week-old guinea pigs from the virus-free colony were anesthetized with ether, a portion of the right submaxillary gland was removed for sectioning, and 0.2 ml of virus inoculated subcutaneously into the area of the left submaxillary gland. At 13 and 22 days, 2 animals

were sacrificed and the left gland was removed. Blood specimens were obtained before inoculation and at sacrifice. Preinoculation submaxillary gland biopsies were uniformly negative, while post-inoculation sections showed characteristic salivary gland disease histopathology, varying in intensity from scattered foci of mononuclear infiltration with few inclusions to more widespread infiltration with moderate numbers of inclusions, several ducts containing 5 to 8 inclusions in a single cross section. Virus was recovered from all 4 glands in tissue culture either on primary isolation or after one blind passage. Both animals sacrificed at 22 days demonstrated CF antibody responses, the titer increasing from $<1:8$ to 1:8 in one animal, and from $<1:8$ to 1:64 in the other. Neutralizing antibody in the latter animal rose from $<1:5$ to 1:40.

Two guinea pigs of the same age and strain as the above animals were inoculated intracerebrally with 0.05 ml of the tissue culture virus and were observed daily for fever and symptoms. Fever was noted in 48 hours and typical symptoms of weakness, lack of coordination, and convulsive movement were evident in 4 days, accompanied by a marked fall in temperature; the animals were sacrificed when moribund, on the 4th and 5th day respectively. Brain sections of both animals revealed marked mononuclear infiltration of the meninges, with typical intranuclear inclusions in mononuclear cells. A suspension of meninges and superficial brain tissue from one animal produced typical cytopathic changes in 6 days after inoculation into tissue cultures. Neither animal had detectable CF antibody in the preinoculation serum specimen.

Additional isolation attempts. In the course of serial passages of the 2 strains of salivary gland virus in guinea pigs, 12 suspensions of submaxillary glands with inclusions were inoculated into explant or trypsin-dispersed tissue cultures of guinea pig embryo muscle. Ten produced cytopathic effects identical with those seen with the strain 1 tissue culture line, with incubation periods of 3 to 10 days; at least 1 subsequent serial passage was possible with 5 of the 7 isolates so tested.

One of the strain 2 isolates has been carried through 8 serial passages with findings completely comparable to those with strain 1; much difficulty was encountered in maintaining the virus during the first 5 passages, but at the 6th passage the incubation period shortened, and the virus has since passed readily. Strain 2 was not distinguishable from strain 1 by CF or reciprocal tissue culture neutralization tests.

No cytopathogenic effects were produced by one suspension of glands which did not contain demonstrable inclusions.

Spontaneous degeneration of tissue cultures of infected salivary glands. Since the human salivary gland virus has been recovered from spontaneously degenerating tissue cultures of human adenoids(3), attempts were made to recover guinea pig virus by cultivating salivary gland tissue. In two experiments, explant cultures were prepared from inclusion-containing submaxillary glands removed on the 17th and 21st day after animal inoculation with guinea pig passage virus. The first of these represented portions of the glands used in suspension to initiate the tissue culture passage line of strain 1. Both sets of cultures grew well, producing primarily fibroblastic outgrowth. On the 28th and 29th days, respectively, cytopathic changes developed which were identical with those produced by infected gland suspensions and tissue culture passage virus. Both sets of cultures produced complement-fixing antigen.

Discussion. The study presented here has indicated that the behavior of the guinea pig salivary gland virus in tissue culture is strikingly similar to that of the human salivary gland virus(2-4). The cytopathic effects of the agents are very similar, and are produced only in fibroblasts. In early passages, the viruses produced effects only after a prolonged incubation period, the rate of progression of cytopathic effects was slow, and the virus could be passed with ease only by using ground cells in the inoculum. In later passages the virus was better adapted to growth in culture and could be passed with cell-free fluids. These similarities suggest that the guinea pig agent would be a useful model in studies designed to anticipate the natural history of human salivary gland virus infection.

The successful passage of the guinea pig

virus in tube cultures, in contrast to the unsuccessful attempts of Andrewes with suspended fragment cultures(5), perhaps is directly comparable to the experience of Weller with varicella virus. Inoculation of tissue fragments with varicella or herpes zoster materials resulted in inclusion body formation which could not be reproduced on serial passage(13); however, using tube cultures and inocula containing cells, Weller was able to carry the viruses in serial passage(14).

Summary. The guinea pig submaxillary gland virus has been successfully propagated in tissue culture, one strain having been carried through 22 serial passages. The tissue culture passage virus produced characteristic intranuclear inclusion bodies in tissue cultures, demonstrated complement fixing and neutralizing antibody responses in guinea pigs infected with animal passage virus, and produced characteristic disease and histopathology in susceptible guinea pigs. Commercial complement consistently contained high titer CF antibody.

It is a pleasure to acknowledge the assistance of Mr. Horace C. Turner, who performed the complement fixation tests.

1. Smith, M. G., PROC. SOC. EXP. BIOL. AND MED., 1954, v86, 435.

2. Smith, M. G., *ibid.*, 1956, v92, 424.

3. Rowe, W. P., Hartley, J. W., Waterman, S., Turner, H. C., and Huebner, R. J., *ibid.*, 1956, v92, 418.

4. Weller, T. H., Macauley, J. C., Craig, J. M., and Wirth, P., *ibid.*, 1957, v94, 4.

5. Andrewes, C. H., *Brit. J. Exp. Path.*, 1930, v11, 23.

6. Enders, J. F., PROC. SOC. EXP. BIOL. AND MED., 1953, v82, 100.

7. Rowe, W. P., Huebner, R. J., Gilmore, L. K., Parrott, R. H., and Ward, T. G., *ibid.*, 1953, v84, 570.

8. Eagle, H., *J. Exp. Med.*, 1955, v102, 37.

9. Bengtson, I. A., *Pub. Health Rep.*, 1944, v59, 402.

10. Beeman, E. A., Huebner, R. J., and Cole, R. M., *Am. J. Hyg.*, 1952, v55, 83.

11. Rowe, W. P., Huebner, R. J., Hartley, J. W., Ward, T. G., and Parrott, R. H., *ibid.*, 1955, v61, 197.

12. Cole, R., and Kuttner, A. G., *J. Exp. Med.*, 1926, v44, 855.

13. Weller, T. H., and Stoddard, M. B., *J. Immunol.*, 1952, v68, 311.

14. Weller, T. H., PROC. SOC. EXP. BIOL. AND MED., 1953, v83, 340.

Received August 9, 1957. P.S.E.B.M., 1957, v96.

Differential Photoreactivation of *Escherichia coli* after Exposure to 2650 and 2250 A Ultraviolet.* (23456)

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The authors have reported(1) that the fraction of the mutagenic and lethal effects of ultraviolet (UV) radiation in *Escherichia coli* cells that is photoreactivable is the same after exposure to 6 wave lengths ranging from 2378 to 2967 A. The fraction of the effect that was photoreactivable was significantly greater for mutation than for inactivation. Extension of these studies to 2250 and 2180 A has revealed some interesting differences in the response to photoreactivating (PR) light and in the response of different strains of *E. coli*.

Inactivation and mutation to streptomycin independence in *E. coli* SD-4 was studied initially. *E. coli* SD-4 is particularly well suited for tests of photoreactivation of mutations since a linear mutation-dose relation is obtained(1,2). The methods of preparation of cells for irradiation and of exposure to UV and PR light have been described(1). Muta-

* This work was supported by Cornell University and the U. S. Atomic Energy Commission through contract with the University.

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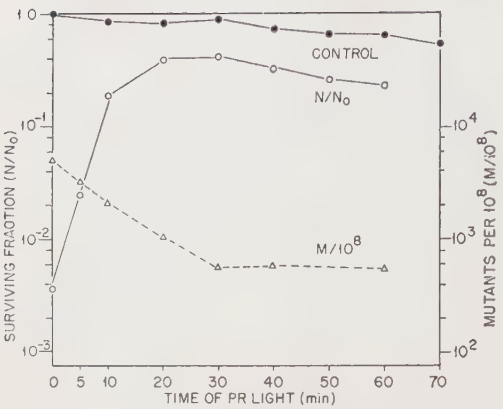

FIG. 1. Survival of *Escherichia coli* strain SD-4 after irradiation with 2650 A ultraviolet.

tion and growth assay methods were similar to those of Anderson(2). For photoreactivation, the samples were placed in small Petri dishes and exposed at 37°C at a distance of 20 cm to the radiation from an AH-5 250-watt mercury arc lamp. The glass envelope was not removed from the lamp and the radiation was filtered through a 5-cm layer of 10% $CuSO_4$ to absorb infrared radiation.

Results. Typical photoreactivation curves of survival ratio and mutants per 10^8 surviving cells are shown in Fig. 1 for SD-4 cells exposed to 2652 A ultraviolet. Similar curves are obtained for 5 other wave lengths between 2378 and 2967 A. The photoreactivation curve for mutation is similar to that for inactivation with an approximately exponential decrease in mutants per 10^8 reaching a minimum (maximum photoreactivation) at about 25 to 30 min exposure, after which the number remains essentially constant.

After exposure of SD-4 to 2250 UV, a quite different response to PR light is observed (Fig. 2). The survival ratio increases initially after exposure to PR light until a maximum is reached at 10 min exposure. Then there is an approximately exponential decrease in survival ratio during continued exposure of the cells to PR light. This decrease in survival ratio is significantly steeper in slope than that experienced by the control cells during exposure to PR light and has been observed repeatedly after exposure to 2250 and 2180 A UV. It appears that these shorter wave lengths in some manner sensitize the SD-4 cells to PR light that, under our conditions, includes wave lengths in the range of about 3100 A up through the visible spectrum. No effort was made to determine which wave lengths in this region were effective in producing this post-UV inactivation. The photoreactivation curve of mutants per 10^8 surviving cells again is quite similar to that for inactivation, with a minimum at 10 min exposure corresponding to the maximum for photoreactivation of the survival ratio. The mutants/10^8 survivors may increase with continued exposure to PR light, but this is not certain.

It appears that the magnitude of photoreactivation of survival ratio after 2250 A UV is actually less than after exposure to 2652 A ultraviolet. The 2250 A photoreactivation survival curve is however a resultant of 2 opposing processes, photoreactivation of UV-inactivated cells and inactivation of survivors of the UV exposure by the PR light. No attempt has yet been made to quantitate these opposing effects of PR light but it is obvious that the actual maximum of photoreactivation is greater than that indicated by the peak of the curve in Fig. 2, and may approach in magnitude that exhibited by cells after exposure to longer UV wave lengths.

The response to PR light of a number of other strains of *E. coli* was studied to deter-

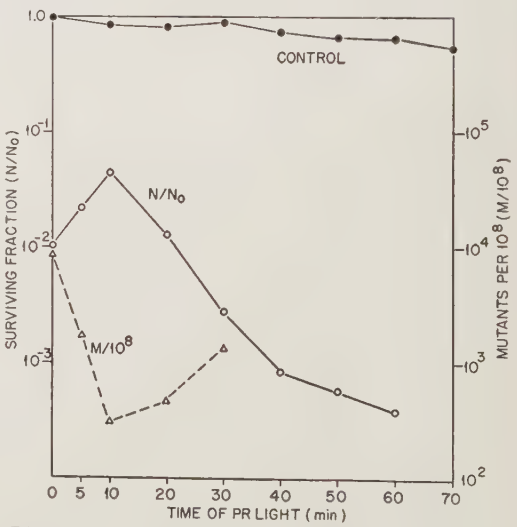

FIG. 2. Survival of *Escherichia coli* strain SD-4 after 2250 A ultraviolet.

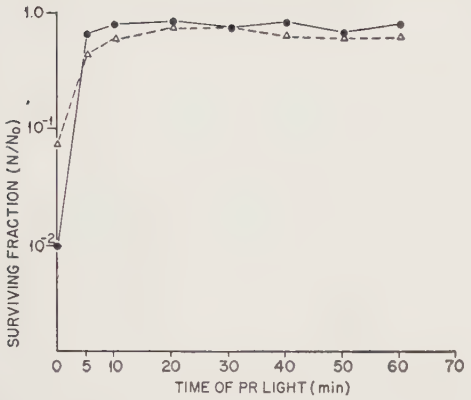

FIG. 3. Survival of *Escherichia coli* strain 42-24 after 2650 and 2250 A ultraviolet.

mine if the behavior exhibited by *E. coli* SD-4 was unique. SD-4 is a streptomycin-dependent mutant of *E. coli* B/r(3), the radiation-resistant mutant of *E. coli* strain B isolated by Witkin(4). *E. coli* strain 82/r is a purine-requiring auxotrophic mutant of *E. coli* B/r that was also employed by Anderson(2) in his studies of X-ray-induced mutations. Survival of B/r and 82/r behaves quite similarly to survival of SD-4 exposed to PR light after 2250 and 2652 A UV. Induced back-mutations in 82/r parallel the survival ratio in response to PR light, but it is known(1) that the apparent rate of mutation in 82/r is subject to other influences than UV dose.

A quite different response to PR light was noted after exposure of *E. coli* strain 42-24 to 2250 A ultraviolet (Fig. 3). Strain 42-24 is a serine-glycine-requiring auxotroph obtained from Dr. R. Girolami and used by him in studies of X-ray-induced back-mutations in which a linear mutation-X-ray dose response curve was observed (personal communication). With strain 42-24, almost identical photoreactivation of inactivation is observed after exposure to 2652 and 2250 A UV. There is no sensitization to PR light after exposure to 2250 A UV. Strain 42-24 also differs from SD-4 in that the maximum of photoreactivation is reached more quickly and the slight decrease in survival ratio observed with SD-4 control and 2650 A-irradiated cells seems to be absent during the 60-min exposure of 42-24 cells to PR light. The mutagenic response of strain 42-24 to UV is so small that statistically accurate back-mutation data have not yet been obtained.

Unfortunately, the origin of strain 42-24, which was added to the culture collection of the Biology Division of the Oak Ridge National Laboratory by the late Dr. E. H. Anderson, is unknown. However, judging from its growth characteristics, radiation response, and motility, we are quite certain that it is not a derivative of *E. coli* B or B/r.

Conclusion. Our data establish that photoreactivation of both inactivation and mutation occurs after exposure of *Escherichia coli* SD-4 to 2250 and 2180 A UV. The magnitude of the photoreactivation after exposure to short-wave-length ultraviolet in *E. coli* B/r and its derivatives is confounded with a short-wave-length UV sensitization to inactivation by the PR light itself. The quite different response to photoreactivating light exhibited by *E. coli* strain 42-24, presumably not derived from *E. coli* B/r, suggests that different strains may differ in response to UV and PR light. It would seem prudent, therefore, to study a number of unrelated strains before generalizations are made concerning mechanisms of radiation action in bacteria.

1. Zelle, M. R., Ogg, J. E., and Hollaender, A., *J. Bacteriol.,* in press.

2. Anderson, E. H., *Proc. Natl. Acad. Sci. U. S.,* 1951, v37, 340.

3. Demerec, M., *Genetics,* 1951, v36, 585.

4. Witkin, E. M., *Proc. Nat. Acad. Sci. U. S.,* 1946, v32, 59.

Received September 9, 1957. P.S.E.B.M., 1957, v96.

Effects of Injection of Opaque Media for Selective Angiocardiography in the Dog's Heart.* (23457)

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Injection of radiopaque material into specific areas of the human heart has become increasingly popular with the increasing use of diagnostic angiocardiography. Surgical therapy is frequently based upon anatomical and functional diagnoses secured in this way. Initially such opaque media were injected by hand syringe with a pressure of approximately 60 lbs./sq. inch or 3,000 mm mercury. Mechanical and compressed air syringes are now being used which inject these materials with a pressure of 360-600 lb./sq. inch. The heart could conceivably be traumatized by such an injection. The usual cardiac dynamics could be upset by such an injection, yielding misleading diagnoses and inadequate or unnecessary surgery. We thought it worthwhile to look into this problem.

Materials and methods. A standard radiopaque contrast material was injected into various chambers and great vessels on both right and left sides of the dog's heart while pressures were being recorded in 2 of the other chambers. Electrocardiographic tracings were obtained at the same time. Thirty-eight mongrel dogs weighing *7* to *18* kg were used in 68 experiments. They were anesthetized with intravenous nembutal. The *jugular* and *femoral* veins were used for insertion of #9 Cournaud cardiac catheters on the right side and the *carotid* and femoral arteries used for the left side of the heart. One cc/kg dog doses of the contrast agent was injected by a compressed air syringe(1) at 6-8 kg/cm^2 pressure. The resultant intracardiac pressures were transmitted through P 23 D Statham strain gauges and recorded on an Offner galvanometer system. In those instances where more than one injection was made into the dog, the next injection was not started until demonstrable effect of the preceding injection had disappeared. Because some of these animals seemed to be developing gross lesions in the heart, the next two procedures were performed. In 16 dogs the only procedure was a single injection of 50% Miokon. The catheter was then rapidly withdrawn. These animals were sacrificed 3 days later and the heart and great vessels were closely examined for evidence of recently acquired disease. In 2 dogs the only procedure performed was insertion of a #9 cardiac catheter into the right atrium and right ventricle respectively where it remained 45 minutes. Following this, the catheter was withdrawn and these dogs were sacrificed 3 days later. Their hearts were obviously diseased.

Results. Table I contains a summary of results of the first 48 experiments in which radiopaque material was injected into a vessel or cardiac chamber and the effect on the electrocardiogram and intracardiac pressures was observed. Ventricular extrasystoles frequently occurred with concomitant alteration in right ventricular and systemic pressures. Such an effect is illustrated in Fig. 1. This shows a reduced right ventricular pressure and ineffectual aortic pulse wave on the second, fourth and sixth systoles after injection. When injections were made into the inferior vena cava or aorta, these alterations were not observed.

In the first group of 31 experiments referred to above, the same dog was used for

TABLE I. Electrical and Pressure Changes Related to Site of Injection of Opaque Media.

Site	No. inj.	No. with abnormal ECG	No. with early pressure drop
Superior vena cava	5	3	3
Inferior " "	5	0	0
Right atrium	6	4	3
" ventricle	14	13	10
Pulmonary artery	5	2	2
Left atrium	1	1	1
" ventricle	6	5	4
Aorta	6	0	0

* Supported by funds from Los Angeles County Heart Assn.

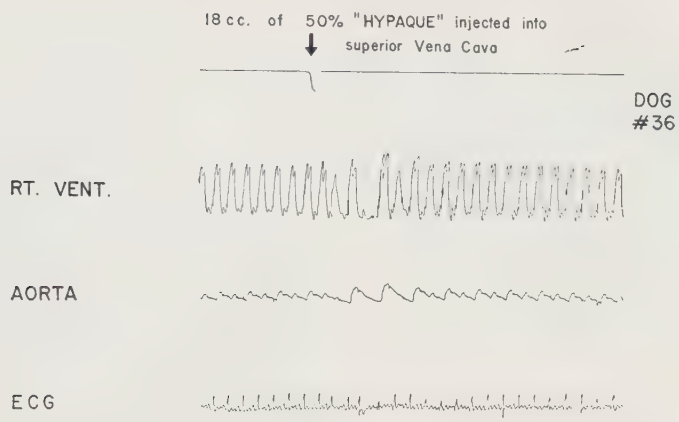

FIG. 1. Effect of inj. of opaque media on pressures and electrocardiogram.

more than one experiment. As a result, the catheters remained in the heart and great vessels for several hours. Of the 13 dogs in these experiments, 8 dogs had subendocardial hemorrhage and/or thrombosis at post mortem examination. The site and nature of the abnormality are indicated in Table II with reference to the point and numbers of injections. Fig. 2 illustrates the extensive nature of the subendocardial hemorrhage, and Fig. 3 illustrates the nature of the thrombus formation.

In the group of 16 dogs in which only the radiopaque material was injected and the catheter promptly withdrawn, no evidence of intracardiac disease was found. Thus it would appear that injection of radiopaque material under these pressures does not *per se* cause intracardiac pathology.

On the other hand, intracardiac thrombosis and hemorrhage *were found* in the 2 dogs in which the cardiac catheter remained in the heart for 45 minutes. The lesions were similar to those found in 8 of the 13 dogs in the first experiment.

Discussion. Selective angiography potentially has a number of advantages over venous angiography(2,3). The purpose of this study was to determine some of the limitations and side effects of rapidly injected, commonly used contrast agents upon the heart. When a radiopaque material is forcibly injected into the right heart, pulmonary artery or superior vena cava, momentary pressure disturbances can occur. These may be attributed in a large part to ventricular extrasystoles associated with the injection. If there were a ventricular septal defect present, opaque material would then proceed from right to left ventricle and thence out the aorta. One might erroneously imply the existence of a right to left interventricular shunt under such conditions.

It seemed reasonable to suppose that intracardiac injection of opaque media under "high" pressure might produce some sort of intracardiac pathology. No such disease was found. Some dogs had subendocardial hemorrhage and thrombi. Our studies indicate that these were related to the presence of the catheter within the heart for periods of over

TABLE II. Dogs in Which Pathological Findings Were Present at Autopsy.*

Dog #	Sites inj.	Sites recorded	Sites with pathology	Types of pathology
3	R.V. 5 times	R.V. & aorta	2 R.A., 2 R.V., mitral valve	Thrombus vegetations
4	R.V. & L.A.	R.V., aorta, carotid	Subendocardium	Hemorrhage
5	L.V.	R.V., L.V.	R.A.	Thrombus
7	R.V.†	R.V., L.V.	2 R.A., L.V.	Hemorrhage
8	P.A.	R.V., L.A.	2 R.A., aortic valve	Thrombus hemorrhage
9	P.A.	L.V., carotid	Aortic valve	Hemorrhage
12	R.A. & L.V.	R.A., fem. art.	R.A.	Thrombus
13	L.V.	R.V., L.V.	R.V., aortic valves	Hemorrhage

* Catheters in heart for a minimum of 1 hr.
† 5% glucose in distilled water inj.

FIG. 2. Cross section of dog #5 heart showing extensive subendocardial hemorrhage.

45 minutes. These complications have not been reported previously.

Conclusions. 1. Subendocardial hemorrhage and intracardiac thrombosis are found when catheters remain in the heart for over 45 minutes. 2. No such lesions could be attributed to "high pressure" intracardiac injections of ordinarily used contrast agents. 3. Disturbed pressure relationships probably due to asynchronous ventricular extrasystoles are frequently seen with injection into the heart, superior cava or pulmonary artery. 4. Such electrical, contractile and pressure disturbances were not noted with injection into the inferior cava or aorta.

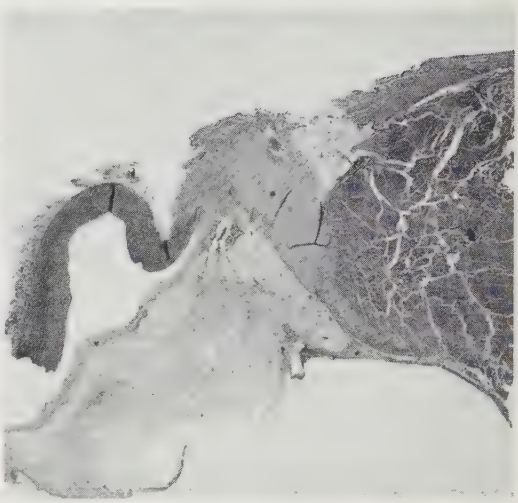

FIG. 3. Cross section of dog #5 heart in region of aortic valves showing a moderate sized thrombus.

1. Gidlund, A., *Acta Radiol., Supplementum,* 1956, 130.

2. Jonsson, G., Brodin, B., and Karnell, J., *Acta Radiol.,* 1949, v32, 486.

3. McFall, R. A., Austin, W. O., Adams, F. H., and O'Loughlin, B. J., *California Med.,* 1957, v86, 162.

Received January 25, 1957. P.S.E.B.M., 1957, v96.

Growth of Several Arthropod-Borne Viruses in Tissue Culture. (23458)

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Diagnostic isolation and identification of arthropod-borne viruses, an antigenically related group with widely variable host range, is difficult for the small laboratory when infections by several types occur in the same area. A single host system susceptible to a majority of the group, conveniently available, easily manipulated, and sensitive to small amounts of virus, is desirable. Tissue culture systems for this group have not been extensively investigated. The group A viruses, eastern (EEE), western (WEE), and Venezuelan (VEE) equine encephalitis viruses have a wide host range and no particular difficulty has been experienced in infecting chick embryo fibroblasts and HeLa cells. Except for West Nile virus, the group B viruses do not produce cytopathic (CP) changes with regularity in the above tissue culture systems (1,2). Reports are lacking on the infection of other tissue culture systems with the group B viruses except for St. Louis encephalitis which produced CP effects on 2 chemically induced rodent tumors and 2 tumors of human origin(3), and Japanese B encephalitis (JBE) and West Nile (WN) which has recently been reported to cause CP effects in Detroit-6 cells (4).

TABLE I. Results of Comparative Titrations of Several Arthropod-Borne Viruses in Mice and Hamster Kidney Cell Culture.

Virus	Strain	Laboratory passage history at start of exp.	Virus derived from infected mouse brain or chick embryo		Virus derived from infected hamster kidney cultures	
			LD_{50} in mice	CPD_{50} in hamster cultures	LD_{50} in mice	CPD_{50} in hamster cultures
EEE	AR-167	*M_2E_1	8.5†	8.5	6.6	7.8
	BCC 6	E_1	9.0	8.0		
WEE	L_2-34a	M_1E_1	7.5	7.3	5.2	7.0
	C 6	E_1	7.5	7.0		
VEE		‡$GP_{13}E_2$	10.2	8.2	6.8	6.8
SLE	904	M_8	8.2	8.5	4.2	4.6
	Hubbard	M_{103}	7.5	7.0		
JBE	Nakayama	M_{97}	6.3	6.5	4.6	4.6
	Ohmori	M_3			4.5	5.2
WN		M_{28}	6.6	8.1	<2.6	3.2
MVE		E_2M_{11}			3.8	3.8
Ilheus		M_{27}			5.8	5.5

* M_2E_1 = Two mouse brain passages followed by one chick embryo passage. † Log_{10}.
‡ GP = Guinea pig brain passage.

The work to be described was an investigation of several tissue culture systems as regards the cytopathic changes produced by arthropod-borne viruses, and a comparison of the sensitivity of tissue culture method with the mouse intracerebral inoculation test.

Materials and methods. Cell suspensions from hamster and guinea pig kidneys, and chick embryos were prepared by overnight trypsinization at refrigerator temperature. Those of dog kidney were trypsinized at 37°C in 30 minute cycles. After 2 washings in Hanks' balanced salt solution, the cells were suspended in growth medium in concentration of 300,000 to 500,000 cells/ml and distributed in 1 ml amounts into glass tubes with bakelite screw caps. These were then incubated in a slanted stationary position at 36°C for 4 to 7 days until ready for use. Kidney cell cultures were grown and maintained in 3 to 5% calf serum, 0.5% lactalbumin hydrolysate in Hanks' balanced salt solution. The medium used for chick embryo fibroblasts consisted of 5% chick embryo extract and 10% calf serum in Hanks' solution. Antibiotics in the proportion of 500 units of penicillin, 250 μg of streptomycin sulfate and 25 units of mycostatin per ml were added to all mediums. Dilutions of virus were made in the respective growth mediums and 0.1 ml volume inoculated per tube. Mice used were 3 weeks old, of the CFW strain. and were inoculated intracerebrally with volumes of 0.03 ml. The viruses used are indicated in Table I.

Results. Dog kidney cells. Cytopathic effects were obtained irregularly in dog kidney cells infected with EEE virus. In comparative titrations in dog kidney cells and intracerebrally in mice the CP changes in the tissue cultures, when they occurred, were always at dilutions more concentrated than those necessary to kill mice. No CP changes were obtained in dog kidney cells infected with St. Louis encephalitis (SLE) virus. Since dog kidney tissue cultures did not satisfy the requirements sought for in a host system with these 2 viruses, no systematic exploration of their sensitivity to the other viruses was made.

Guinea pig kidney and chick embryo cells. Guinea pig kidney and chick embryo cultures and mice were inoculated with 10,000 to 100,000 mouse LD_{50} units of the various viruses. CP changes resulting in complete degeneration of the cells were seen on the second day after inoculation with EEE, WEE, and VEE in both guinea pig kidney and chick embryo cultures. Incomplete degeneration of guinea pig kidney cultures (50%-75% of the cells destroyed) was seen by the 6th day with West

Nile (WN) and Ilheus viruses. No degeneration was seen in the guinea pig cultures by the 6th day with SLE, JBE, or Murray Valley encephalitis (MVE) viruses at which time the fluids were harvested and inoculated into mice. Partial degeneration of the chick embryo cells was seen on the 6th day in those tubes inoculated with WN virus. No changes were seen by the 7th day in the chick embryo cultures inoculated with SLE, JBE, MVE, or Ilheus viruses at which time the fluids were harvested and inoculated into mice. Although all fluids contained virus neither type of culture showed a sufficiently broad spectrum for susceptibility accompanied by CP effects. Therefore, no effort was made to prove or disprove actual increases in virus titer.

Hamster kidney cells. Hamster kidney cultures were inoculated with 10^{-2} or 10^{-3} dilutions of the various viruses. CP changes amounting to complete destruction were produced in these cells in 2 days by EEE, WEE, VEE, and Ilheus viruses. Similar degeneration was seen in 2 to 3 days in cultures inoculated with concentrated dilutions of SLE, JBE, and MVE viruses. West Nile virus produced degeneration in 5 days when a 10^{-3} dilution was used for the inoculum. Uninoculated cultures held during the same period showed no detectable change.

Comparative titrations were made intracerebrally in mice and in hamster kidney tissue cultures using 5 mice and 5 tubes per 10-fold dilution. Appreciably higher titers in mice were found with EEE strain BCC6 and VEE, while the titer of WN was higher in hamster kidney cultures.

Fluids from the tissue culture tubes around the range of the cytopathogenic endpoint were individually inoculated into mice for the detection of virus. In each case virus was recovered from those tubes showing CP effects but not from those which appeared normal, indicating that the infectious dose and cytopathogenic dose were identical. The fluids were harvested from the highest dilutions causing cytopathogenesis in the preceding titrations and a second passage made in hamster kidney tissue cultures. The fluids from these second tissue culture passages were then titrated in mice and in hamster kidney tissue cultures except for a few instances where fluids from the first tissue culture passage were used. The results are shown in Table I.

In general, virus derived from infected tissue culture did not display as high titer as that derived from infected mouse brain. This is probably a reflection of the difference in number of cells per unit volume which were available for infection.

With EEE, WEE, SLE, WN, and the Ohmori strain of JBE the virus derived from infected hamster kidney cultures titered higher in hamster kidney cultures than in mice. The titers in the 2 host systems were the same for VEE, the Nakayama strain of JBE and MVE. With Ilheus virus titers of the first hamster kidney passage material were slightly higher in mice than in tissue cultures.

Using approximately 50 to 100 tissue culture doses of virus, neutralization tests were performed with EEE, WEE, VEE, SLE, and JBE. Equal amounts of the immune serum and virus, and also normal serum and virus, were combined and allowed to stand at room temperature for one hour before inoculation of the tissue cultures. The CP effect was neutralized by the immune serum in each instance except for VEE in which a partial degeneration of the cells took place 2 days after the control tubes were completely degenerated.

Since the passage history of each virus apparently influenced its respective comparative titer, it was thought desirable to attempt direct isolation of virus in hamster kidney tissue culture, comparing this method with intracerebral inoculation of mice and intra-amniotic inoculation of chick embryos. Thirty pheasant and horse brains suspected of being infected with EEE were available for the purpose. Seventeen of these yielded no isolate in any of the 3 host systems. Eleven yielded EEE virus by each of the 3 isolation methods. EEE virus was isolated from one specimen in hamster kidney tissue cultures and in chick embryos but not in mice. Another specimen yielded EEE virus only by chick embryo inoculation, 1 of 6 embryos inoculated succumbing to infection.

To compare the usefulness of hamster kidney cells for isolation of WEE virus, several

tissues from a naturally infected bird, a purple grackle, were inoculated into mice, tissue cultures, and chick embryos. Four mosquito suspensions (*Culex tarsalis*) from which WEE virus had been isolated in 1955 were obtained from Dr. Preston Holden. These arrived in a thawed condition. However it was felt that this fact would allow a more severe test of the sensitivity of the various hosts than if they had remained continuously frozen.

WEE virus was isolated from all of 6 different grackle tissues by inoculation of chick embryos, from 4 of the 6 tissues by inoculation of tissue cultures, and from 3 of the 6 tissues by inoculation of mice.

One of the mosquito suspensions yielded WEE virus by each of the 3 isolation methods. A second suspension yielded virus only in chick embryos, 1 of 6 embryos inoculated dying specifically. No virus could be isolated from the remaining 2 mosquito suspensions by any of the 3 methods.

Heavy brain suspensions produced incomplete degeneration of the hamster kidney cell cultures whether or not virus was present in such suspensions. It was necessary, therefore, to subinoculate into a second set of cultures in order to observe specific typical cytopathogenesis. Mosquito suspensions did not prove toxic to hamster kidney cells and cytopathogenesis progressed in a typical manner when these suspensions contained virus.

Discussion. Hamster kidney tissue cultures proved susceptible to infection with a wide range of arthropod-borne viruses and responded with cytopathogenesis. Those strains of SLE and JBE which had a low number of mouse brain passages titered higher in hamster kidney cultures than in mice, while the reverse was true of those strains having many mouse brain passages. It has been observed in this laboratory that after continued intracerebral passage most of these arthropod-borne viruses lose much of their ability to infect mice by peripheral routes. Thus, laboratory adapted strains and viruses as they are encountered under natural conditions may differ in their relative infectivities for a neural or a visceral tissue. The experience reported here with EEE and WEE viruses indicates that a visceral tissue is more sensitive than brain tissue to "wild" forms of these viruses. The same probably holds true of the SLE-JBE group of viruses in view of the differences obtained with strains of high and low laboratory passage history.

TABLE II. Time Required to Produce Evidence of Infection by Several Arthropod-Borne Viruses in Tissue Cultures and in Mice.

Virus	Strain	Dilution	CP effect in tissue cultures (days)	Death in mice (days)
EEE	BCC 6	10^{-7}	2–3	3– 4
WEE	C 6	10^{-6}	3–4	5– 8
VEE		10^{-7}	2–3	4– 6
SLE	904	"	5–7	6– 8
JBE	Ohmori	10^{-4}	4	8–11
WN		10^{-3}	5	6
MVE		"	5	6– 8
Ilheus		10^{-4}	4	6– 7

Advantages of hamster kidney cultures over mice include in addition to greater sensitivity, less cost, less space for maintenance, and more rapid results. Complete destruction of cultures preceded death of mice receiving equal virus inocula by 1 to 7 days (Table II).

Chick embryos when inoculated by the amniotic route were slightly more sensitive indicators of EEE and WEE virus than were hamster kidney tissue cultures. However, in those cases where the quantity of material available for isolation attempts would limit the choice to a single host system, hamster kidney tissue cultures would be more advantageous than either mice or chick embryos since they display a high sensitivity to all the major arthropod-borne encephalitis viruses which might be encountered in this country.

Summary. Cytopathogenesis was observed in hamster kidney tissue cultures infected with eastern equine, western equine, Venezuelan equine, St. Louis, Japanese B, and Murray Valley encephalitis viruses and by Ilheus and West Nile viruses. The sensitivity of these tissue cultures was equal or superior to that of mice. Guinea pig kidney and chick embryo cultures reacted with complete cytopathic effects only with EEE, WEE, and VEE viruses.

1. Sanders, M., Kiem, I., and Lagunoff, D., *A.M.A. Arch. Path.*, 1953, v56, 148.

2. Scherer, W. F., and Syverton, J. T., *Am. J. Path.*, 1954, v30, 1075.

3. Pollard, M., and Bussell, R. H., PROC. SOC. EXP. BIOL. AND MED., 1952, v80, 574.

4. McCollum, R. W., and Foley, J. F., *ibid.*, 1957, v95, 556.

Received May 27, 1957. P.S.E.B.M., 1957, v96.

Increased Prothrombin Consumption Following Total Body X-Irradiation in Man. (23459)

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In a preliminary investigation of the hematological effects of a single exposure to therapeutic total body x-irradiation in cancer patients, an increase in prothrombin consumption during blood coagulation was observed. The average of postirradiation serum prothrombin time values was significantly elevated over the pretreatment average. The incidence of individual serum prothrombin time prolongations was also significantly increased after irradiation. In addition, it was found that in duplicate tests of serum prothrombin time in which the only known variable was the alternate use of prothrombin-free plasma (Cappel)†, and of purified bovine fibrinogen (Warner-Chilcott)‡, the latter reagent resulted in a significantly more sensitive test in the elevated range.

Materials and methods. Forty-one patients having neoplastic lesions with metastases requiring the use of the total body x-ray therapy according to the Radiation Therapy Committee of M. D. Anderson Hospital were studied. All were ambulatory and, apart from their primary disease, were in general good health and not cachectic. X-ray dosages, delivered in a single total body exposure in each case, (400 kv; 200 cm focal skin distance; 4.1 mm copper half value layer) ranged from 25 r through 150 r in 25 r steps. Dosage in each case depended on medical requirements of the patient. Complete hematological studies were performed daily in a 3- to 5-day baseline period immediately prior to therapy. Similar tests were performed immediately postirradiation and through a 10-day follow-up period before any further radiation therapy was given. Only platelet counts, clotting time, and plasma and serum prothrombin time tests are considered in this report. *Platelet counts* were performed in the Neubauer counting chamber with Rees-Ecker diluent (1). Clotting time tests were made by the 3 tube modified Lee-White procedure(2). Plasma prothrombin time was determined by the Link-Shapiro modification(3) of Quick's one-stage method, using Simplastin (Warner-Chilcott). Serum prothrombin tests were done by a previously reported modification (4,5) of the Quick one-stage method using Simplastin (Warner-Chilcott) and Fibrinogen (Warner-Chilcott). When no end-point was reached within 3 minutes, a reading of 180-plus seconds was recorded. Prothrombin-free plasma (Cappel) was used in place of fibrinogen in duplicate tests in 33 cases.

Results. There was no significant alteration from baseline levels in platelet count, clotting time, or plasma prothrombin time. Also, there was no evidence of relationship of these test results to the serum prothrombin time when the latter was prolonged above average. There was no significant relationship of serum prothrombin time changes to air dose or to integral dose in megagram-roentgens.

Serum prothrombin time. A. *Comparison of pre- and postirradiation averages.* It will be seen in Table I that the posttreatment serum prothrombin time averages, determined by the fibrinogen technic, increased over pretreatment times at the .001 significance level.

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TABLE I. Comparison of Serum Prothrombin Times (SPT) Obtained Using Fibrinogen (F) Technic in Subjects before and after Irradiation.

Radiation dose (r)	No. of cases	Preirrad. SPT avg (sec.)	Postirrad. SPT avg (sec.)	Postirrad. SPT increase	
				No.	%
25	5	41.8	54.3	5	(100)
50	9	56.1	99.8	7	(83)
75	6	56.5	64.4	4	(67)
100	13	47.5	54.0	11	(85)
125	4	40.7	52.2	4	(100)
150	4	35.2	52.2	2	(50)
Total	41	48.1	65.2	33	(80)

Postirradiation averages are statistically significantly larger than preirradiation ones at a level of about .0001. Avg increase of serum prothrombin time (fibrinogen technic) irradiation is 26.2%.

B. *Comparison of frequency of prolonged serum prothrombin times pre- and postirradiation.* Sixty seconds was used as the approximate upper end of the average range(5). The proportion of all readings which fell above this level was determined. The average proportions were: preirradiation = .191; postirradiation = .353; difference = .162. The average difference is statistically significantly larger than 0. ($P<.01$). The number of subjects with negative difference is 4; with zero difference, 20; and with positive difference, 17.

C. *Comparison between serum prothrombin times obtained using prothrombin-free plasma and those obtained using fibrinogen.* In 33 subjects duplicate determinations were made using the fibrinogen and the prothrombin-free plasma methods. The preirradiation average serum prothrombin time with prothrombin-free plasma was 34.8 seconds, 8.9 seconds below the average time with fibrinogen. The postirradiation average time with the plasma reagent was 41.5 seconds, 16.2 seconds below the average time with fibrinogen. The average percentage increase in time with the plasma reagent after x-ray therapy was 16.1%, while the fibrinogen reagent showed 24.3% increase. To test the hypothesis that the fibrinogen technic yields higher values when the prothrombin-free plasma method itself gives elevated results, the correlation between the two technics was analyzed statistically. The hypothesis of no correlation was rejected at a P value of less than .01 in favor of positive correlation. In 24 out of the 33 subjects, the correlation was positive.

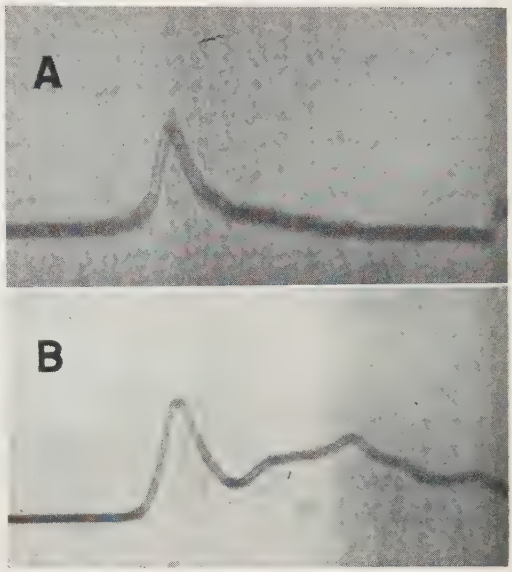

FIG. 1. Electrophoretic pattern of (A) fibrinogen, bovine (Warner-Chilcott) and (B) prothrombin-free plasma (Cappel).

D. *Electrophoretic analysis of fibrinogen (Warner-Chilcott) and of prothrombin-free plasma (Cappel)* is presented in Fig. 1. It will be noted that the former reagent contains fibrinogen in a single pure peak, while the latter reagent contains fibrinogen in a mixture of other plasma protein constituents.

Discussion. Our data indicate the development, in cancer patients, of an alteration in blood coagulation, *i.e.*, increased prothrombin consumption, occurring within 10 days following one dose of therapeutic total body x-irradiation. No reports of similar findings have been found in the radiation hematology literature. In fact, in most of the instances in which prothrombin consumption was studied, reduction was noted at some time following radiation(6,7,8). It is to be noted, however, that the data of Jackson *et al.*(6) indicate that in those of their dogs whose prothrombin consumption was not 100% in the control period (6 out of 8), an increase to 100% occurred at least once in the first 4 days following 600 r whole-body x-irradiation. Also, the mean consumption was in-

TABLE II. Comparison of Serum Prothrombin Times (SPT) Obtained Using Fibrinogen (F) and Prothrombin-Free Plasma (PFP) Technics in the Same Subjects before and after Irradiation.

Radiation dose (r)	No. of cases	Preirradiation SPT avg		Postirradiation SPT avg		Postirradiation SPT increase			
						No.	%	No.	%
		F	PFP	F*	PFP†	F		PFP	
25	3	49.1	38.7	66.6	42.4	3	(100)	2	(67)
50	4	39.6	31.6	49.2	38.7	2	(50)	3	(75)
75	4	50.3	40.8	71.9	55.6	3	(75)	3	(75)
100	13	47.5	37.5	54.0	42.9	11	(85)	11	(85)
125	4	40.7	32.5	52.2	37.3	4	(100)	3	(75)
150	4	35.2	27.5	52.2	32.0	2	(50)	1	(25)
Total	32	43.7	34.8	57.7	41.5	25	(77)	23	(67)

* Avg increase of serum prothrombin time (fibrinogen technic) = 24.3%.
† " " " " " " (prothrombin-free plasma technic) = 16.1%.

creased over baseline on the first and second day postirradiation. Subsequently a marked decrease was found, however, when thrombocytopenia ensued.

Certain limitations of this study should be considered. The number of cases studied was small because of the relative rarity of a therapeutic indication for whole body radiation. For practical reasons the patients studied served as their own controls prior to irradiation and a parallel control series was not run. In addition, the control observation period could not be carried on as long as the postirradiation one. The possibility of a general time-trend effect over the entire experimental period cannot be separated completely from that of radiation effect as the cause of the statistically significant difference in serum prothrombin times before and after irradiation. However, the time-trend differences are not statistically significant by t-test or by a sign test, most of them yielding a P value of about 0.1. Also, clinical experience with the test procedure employed(3,4) did not suggest any time-trend in serial observations heretofore.

Assuming that the observations in this preliminary report are substantiated by similar studies of a large number of cases, an attempt to explain the apparent contradiction of the data in recent pertinent literature is indicated. The frequently noted decrease in prothrombin consumption following irradiation is based primarily on studies of animals, usually after large doses of x-ray at least approaching the lethal range and not always in the immediate period following exposure. Our subjects were humans, receiving a comparatively low exposure and studied promptly. Also, thrombopenia is generally present in animal studies, while it was not found in our series. Court Brown and Abbat noted a similar absence of thrombopenia in their patients receiving one 200 to 425 r skin dose over the spinal column (12). In addition, all of our subjects had metastatic neoplastic disease upon which the radiation may have acted somewhat selectively.

Another difference between our study and those reported in the literature lies in the SPT technic employed. The commonly utilized prothrombin-free plasma may add other protein substances besides fibrinogen to the test system used, as shown by electrophoresis (Fig. 1). That these additional substances might act to mask abnormalities is suggested by the comparative results of serum prothrombin determinations performed by the 2 technics, and by the statistical analysis thereof. The use of pure reagents, when possible, in order to reduce the variables of coagulation test systems is advocated for this reason.

Although the reduction of prothrombin consumption and of serum prothrombin time has been studied extensively by Quick(9) and others, the clinical significance of an alteration in the opposite direction remains to be determined. It has been observed that the average serum prothrombin time in the geriatric age group is higher than that of young adults(4). Dreskin(10) reported a normal range of 25 to 50 seconds but did not account for higher prothrombin consumption evident in a number of normal and diseased subjects

included in his data. Sussman *et al.*(11) reported the average serum prothrombin time in a number of disease states. In many instances this was well above the 30-second level interpreted as the lower limit of normal range. No upper limit was suggested, however.

The reason for the apparent absence of any correlation between onset, magnitude or duration of serum prothrombin time change and either air or integral radiation dose is not readily apparent. Similar lack of quantitative relationship between one large x-ray dose and consequent blood count changes in man has been reported by others(12). Differences in the radiosensitivity of individuals and of neoplasms in our series may be responsible. The selection of the radiation dosage for each case according to medical indications based on the nature and distribution of neoplastic disease may also have helped to obscure any dose effect by introducing bias errors.

It seems possible that alterations in homeostasis produced by disease or by extrinsic agents, such as radiation, could result in imbalances of the many factors involved in the formation of thromboplastin or in the conversion of prothrombin to thrombin. The increase in prothrombin consumption reported here may be an indication of the summation of these alterations, occurring at a time and under conditions of study which have not been carried out previously. This preliminary report is made in the hope that further observations along these lines will aid in the interpretation of the coagulation alteration noted herein.

Summary. 1. Hematological studies were carried out on 41 patients with disseminated neoplastic disease receiving therapeutic whole body x-irradiation. 2. Increased prothrombin consumption, evidenced by an increase in the average serum prothrombin time, and in the incidence of prolonged serum prothrombin times, was found after irradiation. 3. Greater sensitivity resulted when pure fibrinogen was used in place of prothrombin-free plasma in the one-stage serum prothrombin time test. 4. Possible explanations for these findings were discussed.

The author is grateful for the cooperation of the following: Drs. Gilbert Fletcher, John Dillon, Stella Booth, and Mrs. Pauline MacIntyre, Radiology Dept., Anderson Hospital, Houston, Texas; Lt. Col. John E. Pickering, Dept. of Radiobiology, USAF School of Aviation Med., Mr. David Rubenstein, Dept. of Biometrics, for statistical analysis of the data; and Lt. Col. L. J. Milch, Dept. of Pharmacology and Biochemistry, who supervised the electrophoretic protein analyses.

1. Wintrobe, M. M., *Clinical Hematology,* Lea and Febiger, Philadelphia, 1951, 3rd Edit., 239.

2. ———, *ibid.,* 256.

3. Shapiro, S., and Weiner, M., *Coagulation, Thrombosis and Dicumarol,* Medical Press, Brooklyn, 1949, 139.

4. Wald, N., Weiner, M., and Sussman, L. N., *Bull. N. Y. Acad. Med.,* 1952, v28, 609.

5. Weiner, M., and Wald, N., PROC. SOC. EXP. BIOL. AND MED., 1952, v80, 8.

6. Jackson, D. P., Cronkite, E. P., Jacobs, G. J., and Behrens, C. F., *Am. J. Physiol.,* 1952, v169, 208.

7. Ferguson, J. H., Andrews, G. A., and Brucer, M., PROC. SOC. EXP. BIOL. AND MED., 1952, v80, 541.

8. Trum, B. F., and Rust, J. H., *ibid.,* 1953, v82, 347.

9. Quick, A. J., *The Physiology and Pathology of Hemostasis,* 1951, Lea and Febiger, Philadelphia, 1951, 142.

10. Dreskin, D. H., *Am. J. Clin. Path.,* 1952, v22, 140.

11. Sussman, L. N., Cohen, I. B., and Gittler, R., *J.A.M.A.,* 1954, v156, 702.

12. Brown, W. M. C., and Abbat, J. D., *Brit. J. Hem.,* 1955, v1, 75.

Received June 3, 1957. P.S.E.B.M., 1957, v96.

Quantitative Study of Influence of Estrogenic Substances on Serum Lipids of Rats Fed Atherogenic Diet.* (23460)

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The relative infrequency with which clinical manifestations of coronary atherosclerosis are encountered in human females during the reproductive years is well known(1,2). This observation constitutes an unusual example of a natural protective metabolic mechanism which is capable of offsetting atherogenic dietary influences. Clinical observations(3,4) and experimental work with chickens(5,6) and rats(7) have suggested that this protection is related to the effects of estrogens, but the use of estrogens in the treatment of coronary atherosclerosis has been hampered by the feminization which occurs in male patients. Although the mechanisms by which estrogens influence atherosclerosis are not fully understood, clinical(8,9,10) as well as experimental(5,7) studies suggest that suppression of atherosclerosis by estrogens is associated with a higher serum level of phospholipids relative to cholesterol. Since the ratio of cholesterol to phospholipids appears to be associated with development of atherosclerosis in several species under widely diverse circumstances(5,7,11-15) this effect of estrogen on blood lipids may be of primary importance.

The purpose of the present study was 2-fold: first, to obtain additional quantitative information regarding effects of estrogenic substances on serum lipids, and second to see if substances chemically related to estrogens could be demonstrated to influence the serum lipids without causing undesirable changes in the male reproductive organs.

Materials and methods. Animals. In all experiments adult male albino rats weighing between 400-500 g were divided into groups of similar weights and ages. *Diet.* A liquid ration as described by Moskowitz(7) containing 2% protein, 25% fat in the form of corn oil and 2% cholesterol was administered by gastric tube 3 times each day to each animal. During the first week of experimentation the amount of diet administered was gradually increased from 10 ml per feeding up to 12 or 13 ml. The animals were allowed tap water *ad lib.*, but no additional diet. *Estrogen and estrogen analogues.* After the first week of the experiment each animal was given daily (excepting Sundays), subcutaneous injections of the substances dissolved or suspended in sesame oil. *Serum lipids.* Fasting blood samples were obtained from a lateral tail vein of each animal at the beginning of an experiment and at weekly intervals thereafter. For each group equal amounts of blood from each animal were pooled, the serum obtained by centrifugation and total cholesterol and lipid phosphorus determined by standard methods (16,17).

Results. When differing amounts of estradiol benzoate were given, differing effects were obtained (Fig. 1). Among the estradiol-treated animals the largest amount given produced the least elevation of serum cholesterol, but the greatest elevation of phospholipid. Conversely, the least amount of estrogen produced the greatest rise in cholesterol and the least rise in phospholipids. The intermediate amount of estradiol caused an intermediate elevation of both cholesterol and phospholipid. Consequently, the animals which received the smallest amount of estrogen had greatly elevated C/P ratios, while those that received the largest amount had C/P ratios as low or lower than the controls. At all levels of estradiol atrophy of testes, prostate and seminal vesicles occurred.

It should be emphasized that the animals used in these experiments were adult males. The interplay between androgens and estrogens in regard to effects on serum lipids is yet to be fully worked out, but from these results it can be concluded that, at least with adult male rats, one is not justified in speaking of "estrogen effects." The character of the effects depends to a large extent upon how

* This investigation was supported by grants from the Chicago Heart Assn. and from the Nat. Heart Inst. N.I.H., U. S. Public Health Service.

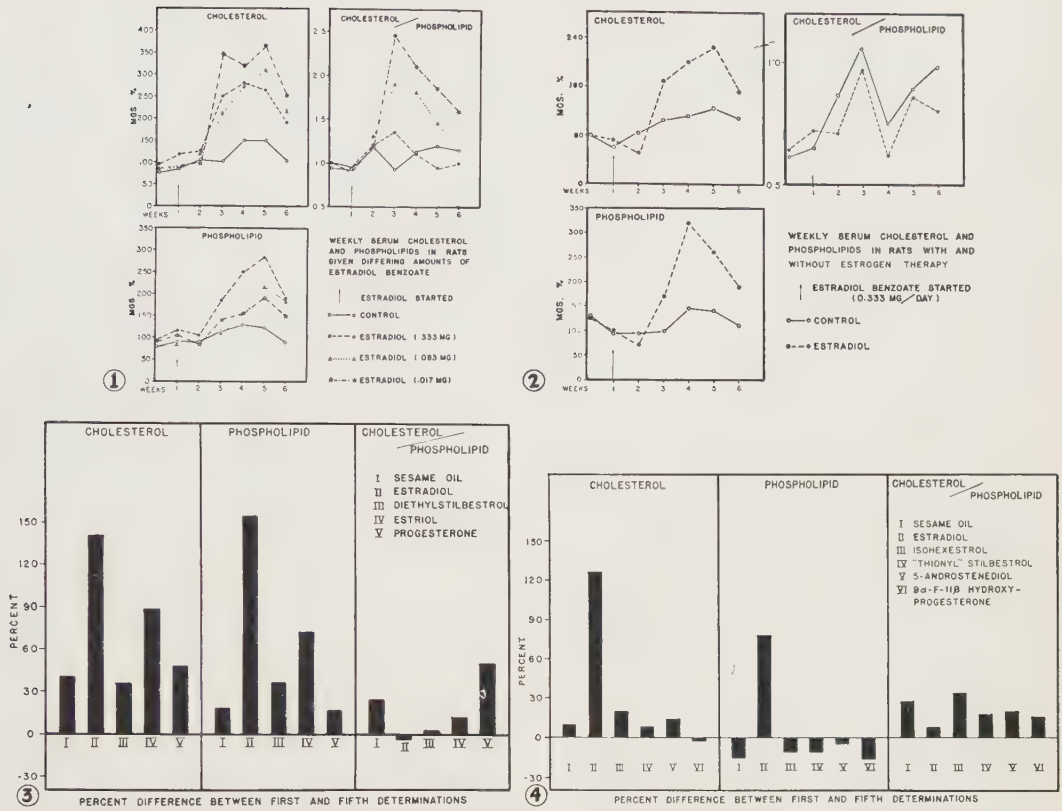

FIG. 1-4.

much estrogen is administered. Because daily administration of 0.333 mg estradiol produced the lowest C/P ratios, this amount was chosen as a reference in the subsequent experiments.

Consequently, in each of the experiments reported here appropriate control groups, one receiving 0.333 mg estradiol benzoate daily and the other receiving only sesame oil were included. When these two groups were compared, the same general pattern of changes in serum lipids with time as that depicted in Fig. 2 was observed in 3 additional experiments. At the beginning of the experiment values for cholesterol, phospholipid and C/P were about the same for treated and control groups. This was a constant observation in these experiments. Both cholesterol and phospholipid levels fell slightly at the end of the first week. By the end of the third week both were elevated, but this elevation was much more marked in the animals which had received estradiol. After reaching a peak a decrease in both cholesterol and phospholipids occurred. The C/P ratio for the group receiving estradiol was lower than that of the control group during the latter part of these 6-week experiments.

With these quantitative relationships in mind a number of natural and synthetic female hormones as well as structurally related compounds were compared. The promotion of the rat's uterine growth by many of these substances has been studied in detail by Huggins and Jensen† (18,19).

Two experiments were designed as shown in Table I.

When the percent difference between values for serum lipids at the end of the fourth week and at the beginning of the experiment were compared (Fig. 3 and 4), both cholesterol and phospholipids were found to have been most

† Some of these compounds were given to us by Dr. Elwood V. Jensen.

TABLE I. Kinds and Amounts of Hormones and Analogues Administered Daily in 2 Experiments. Five animals per series.

Group	"Hormone"	Molecular wt	Amt (mg/day)
I	Control		
II	Estradiol benzoate	271	.333
III	Diethylstilbestrol	268	"
IV	Estriol	287	"
V	Progesterone	314	"
I	Control		
II	Estradiol benzoate	271	.333
III	Isohexestrol	270	"
IV	"Thionyl stilbestrol"*	258	.317
V	5 androstene 3B 17B diol	291	.358
VI	9X fluoro 11B hydroxy-progesterone	351	.433

* 3-(2 thienyl)-4-(e-hydroxy-phenyl)-hexene-3.

elevated in the groups which received estradiol and estriol. The phospholipids were proportionately more elevated than the cholesterol in the group given estradiol, resulting in a fairly constant C/P ratio. Administration of stilbestrol also resulted in maintenance of a fairly constant C/P ratio. To this extent its effect was like estradiol, since the values for the control group rose during this period. Progesterone gave results more like the control group, as did all of the analogues tested.

As can be seen from Table II, the 2 natural estrogens, as well as stilbestrol and isohexestrol caused atrophy of the reproductive glands to about the same degree, while the other estrogen analogues produced atrophy to a lesser degree. Although progesterone apparently produced atrophy in this experiment it did not do so in another.

Atrophy of the male sexual organs occurred with all of the substances studied which had an effect on serum lipids similar to that of estradiol.

Discussion. The reported effects of estrogenic substances on blood lipid concentrations and on atherogenesis are remarkably variable. For example with 1 mg per day of estradiol benzoate for 13 weeks, Pick *et al.*(5) reported suppression of coronary atheromatous lesions in the chicken accompanied by a definite rise in blood lipid phosphorus and reversal of coronary lesions with 1 mg per day for 5 weeks (6). Other workers(20,21) have indicated an increase in atherogenesis and hyperlipemia in this same species when 25 mg pellets of the somewhat less potent diethylstilbestrol were implanted about once a month for varying periods. In the estrogen-treated rat, Moskowitz *et al.*(7) observed a marked rise in both serum cholesterol and lipid phosphorus as compared to controls when the animals were force-fed a high fat diet. In this study 0.333 mg of estradiol per day appeared first to lead to increased susceptibility to coronary atheromatous lesions followed later by suppression or reversal of the lesions. Other results in the rat have been reported recently by Fillios *et al.*(22) who found that 0.02 mg of estradiol injected 2 times per week for 7 weeks increased the serum cholesterol concentration. Under similar experimental conditions estrogen treatment enhanced cardiovascular sudanophilia has been reported in male rats (23).

The results of the present study suggest that at least some of this variation in the effects of estrogenic substances may be related to the dose and period of administration. Similarly the biphasic effect on atherogenesis produced by estradiol in the rat(7) may be explained by a gradually increasing daily effect of the estrogen resulting from multiple sesame oil depots which may superimpose their liberated estradiol.

It will be necessary to find out whether the differing effects of various doses of estrogen on blood lipids and atherogenesis are observed in other species. It is possible that this concept may have broad implications in terms of the natural history of atherogenesis in man. Observations relating estrogenic therapy to blood lipids in human(4,10) as well as in

TABLE II. Average Weights of Reproductive Glands of Rats Given Estrogens and Analogues.

Group	Prostate (g)	Testis (g) R	Testis (g) L
I	1.62	1.50	1.54
II	.73	.33	.36
III	1.01	.31	.32
IV	.97	.34	.35
V	1.03	.58	.60
I	2.69	1.65	1.59
II	1.52	.53	.53
III	1.61	.92	.72
IV	2.62	1.73	1.46
V	2.30	1.14	1.51
VI	1.92	1.34	1.36

other species(24) may need further evaluation in the light of dosage and time effects demonstrated in this study. The tendency for blood lipid values of the rat to decline after several weeks of therapy suggests that an adaptive mechanism may be present which will tend to counteract the early effects of estradiol even when a large dose is administered.

Recent observations(25) which relate lipid phosphorus rise in the human male to estrogenic analogue administration while demonstrating little evidence of estrogenic effect on secondary sex characteristics represent a desirable goal and deserve further evaluation in experimental animals as well as in man.

Summary. 1) In adult male albino rats varying amounts of estradiol, all within a range sufficient to produce testicular atrophy, differed in their effects on serum lipids, the smaller amounts causing marked elevation in serum cholesterol and less elevation in phospholipids than larger amounts. Administration of 0.333 mg estradiol benzoate daily resulted in marked elevation of serum cholesterol and phospholipids within 2 weeks, but the C/P ratio remained as low or lower than that of control animals. Over a 5-week period these values reached a peak, then fell in spite of constant diet and hormonal treatment. 3) Equivalent amounts of estriol daily had an effect on serum lipids similar to that of estradiol. 4) Equimolecular amounts of stilbestrol, progesterone, and a variety of substances chemically related to active estrogens had little acute effect on serum lipids under the conditions studied. 5) This experimental plan may be useful for studying simultaneous effects of other steroids on serum lipids and on the reproductive organs of mammals.

1. Gover, M., and Pennel, M. Y., *Pub. Health Rep.,* 1950, v65, 819.

2. Levy, R. L., Bruenn, H. G., and Kurtz, D., *Am. J. Med. Sci.,* 1934, v187, 376.

3. Wuest, J., Dry, T. J., and Edwards, J. E., *Circulation,* 1953, v7, 801.

4. Rivin, A. U., and Dimitroff, S. P., *ibid.,* 1954, v9, 533.

5. Pick, R., Stamler, J., Rodbard, S., and Katz, L. N., *ibid.,* 1952, v6, 276.

6. ———, *ibid.,* 1952, v6, 858.

7. Moskowitz, M. S., Moskowitz, A. A., Bradford, W. L., and Wissler, R. W., *Arch. Path.,* 1956, v61, 245.

8. Stamler, J., Pick, R., and Katz, L. N., *Ann. N. Y. Acad. Sci.,* 1956, v64, 596.

9. Eilert, M. L., *Metabolism,* 1953, v2, 137.

10. Barr, D. P., *J. Chronic Diseases,* 1953, v1, 63.

11. Gertler, M. M., Garn, S. M., and Lerman, J., *Circulation,* 1950, v2, 205.

12. Kellner, A., Correll, J. W., and Ladd, A. T., *Am. Heart J.,* 1949, v38, 460.

13. Wissler, R. W., Eilert, M. L., Schroeder, M. A., and Cohen, L., *Arch. Path.,* 1954, v57, 333.

14. Steiner, A., Davidson, J. D., and Kendall, F. E., *Am. Heart J.,* 1948, v36, 477.

15. Mann, G. V., Andrus, S. B., McNally, A., and Stare, F. J., *J. Exp. Med.,* 1953, v98, 195.

16. Abell, L. L., Levy, B. B., Brodie, B. B., and Kendall, F. E., *J. Biol. Chem.,* 1952, v195, 357.

17. Fister, H. J., *Lipid Phosphorus, Method* P-37.1, *Manual of Standardized Procedures for Spectrophotometric Chemistry,* N. Y. Standard Scientific Supply Corp. 1950.

18. Huggins, C., and Jensen, E. V., *J. Exp. Med.,* 1955, v102, 335.

19. ———, *ibid.,* 1955, v102, 347.

20. Chaikoff, I. L., Lindsay, S., Lorenz, F. W., and Entenman, C., *J. Exp. Med.,* 1948, v88, 373.

21. Lindsay, S., and Chaikoff, I. L., *Arch. Path.,* 1950, v49, 434.

22. Fillios, L. C., *Endocrinology,* 1957, v60, 22.

23. Fillios, L. C., and Andrus, S. B., *Fed. Proc.,* 1957, v16, 356.

24. Fillios, L. C., and Mann, G. V., *Circulation Res.,* 1956, v4, 406.

25. Robinson, R. W., Cohen, W. D., and Higano, N., *Circulation,* 1956, v14, 489.

Received June 13, 1957. P.S.E.B.M., 1957, v96.

Alteration of Serum Cholesterol by Dietary Fats.* (23461)

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The concentration of cholesterol in the blood serum of man can be reduced through subsistence on a low-fat diet(1-5) or by substitution of certain vegetable oils in the diet (6-11). Recognition of the fact that an elevated serum cholesterol level tends to be related to the development of atherosclerosis has led to widespread interest in the possibility that regulation of the fat content of the diet may help to control or prevent ischemic (coronary) heart disease(4,8,12,13). One result of this attitude is the recent use of corn oil in the diet, sometimes on medical prescription, by persons with hypercholesterolemia or coronary heart disease or both.

Corn oil when fed in large amounts has been repeatedly shown to produce a large depression in the serum cholesterol level(8-11, 13,14). This effect has been attributed to its high content of polyunsaturated fatty acids by some workers(8,9,13,14), but the possibility that this characteristic of corn oil explains only part of the effect is also indicated(11,13, 15,16). Examination of these reports shows that in some cases the subjects had metabolic peculiarities including hypercholesterolemia, obesity and diabetes. In many of the studies synthetic and artificial mixtures instead of natural foods were used as diets, and in practically all cases the experimental fat was isocalorically substituted for carbohydrate or for another fat. Further, in many of the studies the numbers of persons studied did not permit proper statistical analysis.

Accordingly, it was considered desirable to compare several food fats, including corn oil, in regard to the influence on the serum cholesterol, when taken as a simple supplement to the diet in the manner now being used by an appreciable number of patients, and to make such a comparison with enough subjects, relatively homogeneous in type, to allow valid statistical analysis. *Subjects.* The subjects were the 141 volunteers in the laboratory courses in Physiological Chemistry who satisfactorily completed the prescribed dietary regimen for which 159 originally volunteered. Their mean age was 22.8 years (range 20-34). Two of the volunteers were young staff members, 10 were graduate students, 16 were Medical Technology students and 113 were freshmen in the Medical School. These volunteers were organized by the class officers.

Materials and methods. The characteristics and source of the 4 fats employed are given in Table I. In order to make the dietary fat supplements palatable and to disguise their identity the fats were incorporated in "milkshakes." The composition of each milkshake was: fat 57 g (2 oz), fat-free dried milk 30 g, sucrose 10 g, Dariloid K† 0.5 g, a mixture of mono- and distearates of glycerol 0.5 g‡, double strength vanilla extract 1.0 ml, and water to make 300 g. The milkshakes containing the fat of cream each contained 171 ml of 35% whipping cream and were made with 20 g of dried fat-free milk instead of 30 g owing to the milk proteins contained in cream. The taste of the olive oil containing milkshakes was difficult to render acceptable and these were additionally flavored with coffee extract.

Each type of milkshake was prepared by homogenization in bulk quantity, pasteurized and packaged in 300 g amounts in color-coded individual paraffined paper milk cartons by the Dairy Husbandry Dept. of the Univ. of Minnesota. The preparations were kept

* The members of the Class of 1957 in Physiological Chemistry, Medical School, University of Minnesota, were the subjects. These students actively participated in planning of the work and in chemical and statistical analyses which were also independently carried out by the authors.

† A preparation of alginic acids used as an agent for stabilizing emulsions. Obtained from Kelco Co., Chicago, Ill.

‡ An emulsifying agent labeled Atmos 150. Obtained from Atlas Powder Co., Wilmington, Del.

TABLE I. Characteristics of Fats Tested.

Fat	Iodine No.	Linoleic acid, g/100 g fat	Linolenic acid, g/100 g fat
Cream	31.9	1.53	.60
Corn oil	126.7	57.5	.86
Safflower seed oil	144.2	72.0	.48
Olive oil	85.8	9.5	—

These analyses were kindly carried out by Dr. R. T. Holman, Hormel Institute, Austin, Minn. Cream used as 35% whipping cream; corn oil from Clinton Foods, Inc., Clinton, Iowa; edible safflower seed oil from Pacific Vegetable Oil Co., San Francisco, Calif.; olive oil as Gerber's U.S.P. olive oil.

frozen until the day before they were to be dispensed when they were transferred to a cold-room at 4°C.

Each student ingested one milkshake of his assigned code per day for 9 days and recorded on a chart in the laboratory the fact that he had or had not consumed the whole of each day's milkshake. The time-of-day and the rate at which the milkshakes were consumed were entirely self-chosen and varied considerably among the students. No attempt was made to regulate the basal diet of the students beyond indicating to them that they could reduce their consumption of bread and desserts if they were concerned about gain in body weight. The results of serum cholesterol determinations of all persons who failed on 2 or more days to consume a milkshake were excluded from the tabulation of the results. The data of 18 persons were excluded on this basis. Some of the individuals consuming the safflower oil containing supplements complained of diarrhea and dropped out of the study group for this reason. Fasting blood samples, from which the sera were harvested, were obtained by vena puncture just prior to ingestion of the first milkshake and on the day following the consumption of the last milkshake. Body weights were recorded on the same days. Each student made a single determination for total cholesterol content in his own 2 sera samples by the method of Kingsley and Schaffert(17). The analyses of the 2 samples were carried out concurrently, which permitted the colorimeter readings of the initial and final samples to be referred to the same developed cholesterol standard. The same sera were independently analyzed in duplicate for total cholesterol in the Laboratory of Physiological Hygiene by the method of Anderson and Keys(18).

No subject or other person who took part in the analyses or in the final compilation of the results was aware of the identity of the fat in the coded milkshakes until after the selection and summation of the results. The results of 4 analyses by students of initial and final serum samples were excluded because the recorded result departed from the mean initial result for the entire study group by more than 2 standard deviations. The initial and final analyses obtained by 11 other students were discarded because of known laboratory accidents or errors. The number of subjects given in the tables refers in each case to the number of individuals who satisfactorily completed the dietary regimen and on whose serum samples acceptable initial and final cholesterol determinations were obtained.

Results. The results are given in Tables II

TABLE II. Alteration of Serum Cholesterol Contents during Periods of Fat Intake.

Dietary group	No. of persons*	Initial serum cholesterol, mg/100 cc	Deviation of final serum cholesterol from initial value			
			mg/100 cc	%	t	P
Corn oil	A 34	226.7 ± 50.77†	−37.0 ± 6.16‡	−16.3	6.01	<.001
	B 32	205.4 ± 33.72	−23.0 ± 2.84	−11.2	8.10	<.001
Olive oil	A 35	234.0 ± 46.70	−14.2 ± 7.68	− 6.1	1.85	.07
	B 36	197.6 ± 27.11	−10.7 ± 2.32	− 5.4	4.62	<.001
Safflower oil	A 26	220.3 ± 32.84	−22.8 ± 5.92	− 9.9	3.85	<.001
	B 28	201.9 ± 34.55	−18.0 ± 4.56	− 8.9	3.95	<.001
Cream	A 31	228.6 ± 49.23	+ 8.9 ± 7.41	+ 3.9	1.2	.23
	B 32	194.6 ± 29.72	+ 2.8 ± 3.19	+ 1.5	.88	.37

* A = Data obtained by students. B = Data obtained by Laboratory of Physiological Hygiene.

† Mean ± S.D. ‡ Mean ± S.E.

TABLE III. Initial Body Weights and Body Weight Gains.

Dietary group	No. of persons	Initial body wt (mean ± S.D.)	Gain in body wt (mean ± S.E.)	Significance of body wt gains	
				t	P
Corn oil	32*	165.7 ± 25.25	1.28 ± .35	3.66	<.001
Olive oil	36	160.4 ± 20.47	.95 ± .37	2.59	.012
Safflower oil	28	164.8 ± 20.44	2.25 ± .37	6.10	<.001
Cream	32	155.0 ± 24.02	1.15 ± .32	3.60	<.001

* 2 subjects failed to record final wt.

and III. Statistical treatment of the results was made by a method of paired variates.§ This method gives a measure of the significance of the over-all differences between the initial and final observations for the individual subjects in each group.

The students obtained higher values for total serum cholesterol than were observed in the Laboratory of Physiological Hygiene on analysis of the same sera primarily, it is believed, because of differences in methodology in analytical procedures. The method used by the students does not involve hydrolysis of the cholesterol esters whereas the method of Anderson and Keys(18), a modification of the method of Abell *et al.*(19), effects hydrolysis with alkali before application of the Liebermann-Burchard reagent for development of the color. Since cholesterol esters give a higher color intensity than free cholesterol in the Liebermann-Burchard reaction and free cholesterol is used as the standard, a method which does not hydrolyze the esters will necessarily give high values. From Table II the magnitude of this systematic over-estimation in the students' analyses can be computed. In the 8 sets of comparisons (4 groups, each group "initial" and "final"), the students' averages range from 4.0 to 20.3% above those from the Laboratory of Physiological Hygiene, the grand mean discrepancy being 13.1%.

The standard deviations of the means of the student analyses are larger than those of the mean results obtained by the Laboratory of Physiological Hygiene due, undoubtedly, to the lesser technical skill of the students. Nevertheless, owing to the large number of analyses, the same general conclusions, with one possible exception (olive oil group), are indicated by each set of determinations.

The groups ingesting the 3 vegetable oils each exhibited statistically significant decreases of total serum cholesterol while the subjects receiving cream exhibited a slight rise which is not significant statistically. The result obtained with the corn oil group was of greater magnitude than that obtained with the other 2 vegetable oils. The difference in effect produced by corn oil and olive oil appears to be real ($P<0.01$) but the data do not demonstrate a statistically significant difference between the effects produced by corn oil and safflower oil. Safflower oil is the most unsaturated and has the highest linoleic acid content of the oils tested (Table I), and if these characteristics were the sole determinants of the cholesterol depressing action of these vegetable oils, safflower oil should have exhibited a clearly superior effect over that of corn oil. Since this result was not obtained, and because the result was, in fact, in the opposite direction, it appears that the cholesterol lowering qualities of corn oil are not due solely to its content of linoleic acid or degree of unsaturation as expressed by the iodine value.

Each of the 4 groups of students exhibited significant increases in body weight over the 9 days of the experiment, the grand average gain being 1.37 lb or 0.62 kg. This fact indicates that the subjects did not reduce their caloric intake from other foods by an amount

§ $t = \sqrt{\frac{(N-1)\,(\Sigma d)^2}{N\Sigma d^2 - (\Sigma d)^2}}$ where "N" is the number of cases and "d" is the difference between the initial and final observations for each subject. The calculated values of "t" are recorded in the tables in terms of probability that the results are due to chance. The calculations were independently checked by the authors.

equal to that of the milkshakes. Nevertheless, some reduction of intake of ordinary food of the diet must have occurred with a majority of the subjects because the weight gain, with the exception of the safflower oil group, was not as great as would have resulted had the milkshake calories been added to those of an unreduced pre-experimental diet. A gain of 1 kg from simple overeating represents tissues having a value of about 6180 calories(20). Since the caloric content of the milkshakes over the 9-day period was 6157 calories the subjects would all have gained about 1 kg had they not reduced their intake of ordinary food. Only in the case of the safflower oil group was the weight gain of this magnitude. The average weight gain of the other 3 groups was 1.1 lb or 0.5 kg. Hence it is indicated that these students stored on an average 3090 calories. Since the milkshakes provided 6157 cals. a total of 3067 cals., or 340 cals. per day, of ordinary food of the diet was supplanted by the milkshakes. It is not possible to determine what ordinary food items were involved in this dietary substitution but it must be assumed that a part of the reduction in the ordinary diet was at the expense of beef and dairy fats since these are prominent in the usual diet and are most easily omitted at the table. These considerations then lead to the point that a part of the reduction of serum cholesterol concentration in the subjects in the corn oil and olive oil groups may have been due to reduced intake of saturated fats. The same consideration also suggests that the cholesterol lowering in the safflower oil group might have been of a somewhat greater degree had the individuals in this group reduced their intake of ordinary food fat to the same extent as those of the other groups.

It would appear that the serum cholesterol changes observed may have been the result of the following factors: 1) addition to the diet of 57 g per day of the experimental fat, 2) removal of ordinary diet fat, mainly of the saturated type, 3) slight positive calorie balance with attendant requirement of extra fat transport in the body to fat stores, 4) operation of the factor associated with unsaturation of fatty acids in the experimenal fat, and 5) operation of the "X" factor in corn oil.

While evaluation of the contribution of each of the several factors listed above to the cholesterol change cannot be precise, the findings as to net effects of the several fats used as supplements show what may be expected with persons using such supplements with a view to serum cholesterol control. Corn oil is indicated to be the fat of preference but without any clear margin over safflower oil. Since the body weight response was almost identical in the groups ingesting butterfat, olive oil and corn oil, the results with these fats raise no question of full comparability as is the case with safflower oil.

Summary. Serum total cholesterol concentration was measured in 122 young men and 19 young women before and after 9 days during which each person ingested daily 57 g of corn oil, olive oil, safflower oil or butterfat. The subjects were instructed to follow their usual diets during this period and body weight measurements indicated that the experimental fats did not supplant an equal quantity of ordinary diet calories. The subjects who ingested butterfat showed a slight but statistically insignificant rise in the serum cholesterol while those who ingested the other fats exhibited a statistically significant decrease, averaging 23.0 $\pm$ 2.8 mg per 100 ml with corn oil, 18.0 $\pm$ 4.6 with safflower oil and 10.7 $\pm$ 2.3 with olive oil. Compared with the safflower oil the corn oil was more saturated (iodine value 126.7 vs. 144.2) and contained less linoleic acid (57.5 vs. 72.0%), so it is concluded that the cholesterol depressant action of the corn oil was not fully accounted for by its degree of unsaturation or content of "essential" fatty acid. Since the subjects in all groups gained some weight, it appears that at least some effects of the 3 vegetable oils tested can be obtained without exact isocalorie substitution in a normal diet.

1. Kempner, W., *Ann. Int. Med.,* 1949, v31, 821.

2. Keys, A., *Circulation,* 1951, v5, 115.

3. Groen, J., Tjiong, B. K., Kamminga, C. E., and Willebrands, A. F., *Voeding,* 1952, v13, 556.

4. Keys, A., *J. Mt. Sinai Hosp.,* 1953, v20, 118.

5. Mayer, G. A., Connell, W. F., DeWolfe, M. S., Beveridge, J. M. R., *Am. J. Clin. Nutrition,* 1954, v2, 316.

6. Kinsell, L. W., Michaels, G. D., Partridge, J. W., Boling, L. A., Balch, H. E., and Cochrane, G. C., *J Clin. Nutrition,* 1953, v1, 224.

7. Ahrens, E. H., Jr., Blankenhorn, D. H., and Tsaltas, T. T., PROC. SOC. EXP. BIOL. AND MED., 1954, v86, 872.

8. Bronte-Stewart, B., Antonis, A., Eales, L., and Brock, J. F., *Lancet,* 1956, v1, 521.

9. Ahrens, E. H., Jr., Tsaltas, T. T., Hirsch, J., and Insull, W., Jr., *J. Clin. Invest.,* 1955, v34, 918.

10. Beveridge, J. M. R., Connell, W. F., and Mayer, G. A., *Circulation,* 1956, v14, 484.

11. Keys, A., Anderson, J. T., and Grande, F., *Lancet,* 1957, v1, 66.

12. Keys, A., *J. Chronic Dis.,* 1956, v4, 364.

13. Malmros, H., and Wigand, G., *Lancet,* 1957, v11, 1.

14. Ahrens, E. H., Jr., Insull, W., Jr., Blomstrand, R., Hirsch, J., Tsaltas, T. T., and Peterson, M. L., *ibid.,* 1957, v1, 943.

15. Anderson, J. T., Keys, A., and Grande, F., *J. Nutrition,* 1957, v62, 421.

16. Beveridge, J. M. R., Connell, W. F., and Mayer, G. A., *Fed. Proc.,* 1957, v16, 11.

17. Kingsley, G. R., and Schaffert, R. R., *J. Biol. Chem.,* 1949, v180, 315.

18. Anderson, J. T., and Keys, A., *Clin. Chemistry,* 1956, v2, 145.

19. Abell, L. L., Levy, B. B., Brodie, B. B., and Kendall, F. E., *J. Biol. Chem.,* 1952, v195, 357.

20. Keys, A., Anderson, J., and Brozek, J., *Metabolism,* 1955, v4, 427.

Received June 21, 1957. P.S.E.B.M., 1957, v96.

Procedure for Transplantation of Dog Kidney without Interruption of Renal Blood Supply.*† (23462)

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Kidneys transplanted from one dog into another are usually rejected within five to ten days(1). A characteristic pathology develops in these tissues and it is generally accepted that this rejection is mediated through an immunological reaction(2). The operative procedure previously employed for transplantation requires an interruption in the blood supply for at least 20 minutes. The contribution of the ischemic period to the development of the subsequent metabolic and pathologic states is unknown; however, it seems likely that it may produce non-specific damage to the renal tissues. We have devised a technic whereby the kidney can be transplanted without interruption of its blood supply, thus permitting study of the rejection process without the complicating factor of ischemia.

Methods. In principle, the new procedure takes advantage of the fact that the aorta and vena cava form a "T" connection with their respective renal vessels. This structural arrangement permits perfusion of the renal vessel from below during the time when the upper section of the aorta or vena cava must be occluded for the vascular suturing. For example, in the arterial system the blood may flow into the renal vessel either from above as it normally does or from below the point of origin of the artery on the aorta. In our procedure, the accessory circulation provides arterial blood for the kidney through the aorta via a connection through a plastic tube with the host's carotid artery. This blood enters the aorta below the point of origin of the renal vessel. The same principle is used to return the renal venous blood through the vena cava below the point of origin of the renal vein, and back through the external jugular to the donor's heart. The donor animal is prepared by dissection of the left kidney, its ureter and the renal vessels. The aorta and vena cava are also freed for a distance of 1½ inches above and 1½ inches below the renal vessels. All of the major abdominal vessels except those referred to above,

* Supported in part by the Josiah Macy, Jr. Fn.

† The authors gratefully acknowledge the assistance of Genevieve Edwards, R. N.

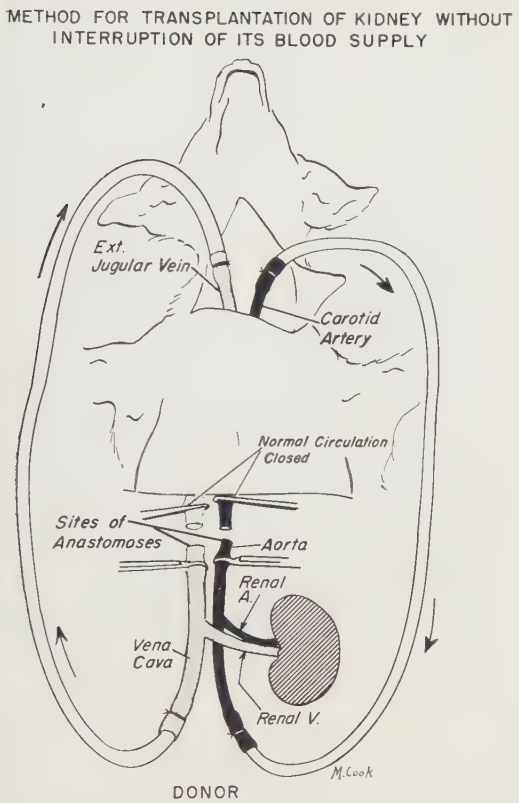

FIG. 1. This diagram illustrates state of the donor just before kidney is transferred to the host. Note sites of anastomoses which will be connected to iliac vessels of the host.

are tied and divided. The carotid artery and jugular vein are also isolated and, as mentioned above, these vessels are employed to provide the accessory circulation. A polyethylene tube about 2½ feet long is filled with saline and inserted into the carotid artery. The opposite end of this tube is put into the aorta below the renal artery. Clamps are applied until a flow of blood through the tubes is desired. A similar procedure is used for the venous system to connect the vena cava distal to the renal vein with the donor's external jugular vein. Clotting in the tube is controlled by intravenous administration of heparin. During the manipulations described above, the normal channels for renal blood flow are intact. The clamp on the tubing is now removed so that blood flows from the carotid artery into the aorta and from there through the renal artery to supply the kidney. At the same time that the clamp is removed from the tubing, another clamp is placed on the aorta above the renal artery so that the normal route of blood flow is blocked. The venous system is treated in a similar manner. The venous blood now flows from the renal vein into the vena cava and then via the plastic tubing into the external jugular vein of the donor. The aorta and vena cava are tied and divided rostral to the renal vessels. The kidney can be lifted from its bed and moved to an adjoining operating table without interruption in its blood supply. Fig. 1 illustrates the conditions prevailing just prior to the time the donor kidney is removed from its normal position.

The host has already been prepared on the adjoining operating table. Its iliac vessels on the side nearest the donor are isolated, clamped and divided. The aorta and vena cava are anastomosed to the iliac artery and vein, respectively. The most difficult part of the surgery results from the need to adjust the diameter of the cut end of the vessels so that there will be no subsequent constriction at the suture line. The circumference of the smaller iliac vessels can usually be enlarged by cutting it at an angle. In this way it is possible to match it to the larger aorta or vena cava.

When the vascular anastomoses are completed, the clamps on the host's vessels are removed to allow the host's blood to pass through the kidney. At the very moment that these clamps are released, another set is applied to the tubing, blocking the donor blood flow to the kidney. Thus, at no time is there an interruption in the blood supply to the kidney nor an interchange of donor and host blood.

The transplanted kidney is placed in the abdominal cavity of the host and its ureter is exteriorized through a stab wound. Urine can be collected from the isolated ureter.

The kidney has a continuous blood supply throughout the procedure. Pulsations from the renal artery can be directly observed during the operation. The capsule retains its normal pink color, and some of its vessels are noted to bleed freely. The ureter remains so well vascularized that the blood loss from its cut end must be controlled. Equally im-

portant, urine is observed to flow throughout the operation, and continues without interruption when the host's blood is started through the kidney.

Twenty-four operations have been performed. One of the animals failed to produce urine and was discarded within 4 hours following surgery. Twenty-three of the operations were acutely successful. However, 10 of these kidneys failed to function for more than one day due to the formation of a thrombus in the renal vein or artery. Thirteen of the transplants continued to secrete urine for periods of one to 8 days, at which times the kidneys were removed.

The lack of ischemia during the transplantation operation does not confer a marked prolongation of function to the kidney. However, there appear to be some benefits from transplantation without interruption in the blood supply. The tissue in Fig. 2 illustrates the extent of the rejection process six days after transplantation. The cellular infiltration is not prominent. On the other hand, a kidney which has been subjected to a period of ischemia during the operation (Fig. 3) shows a marked infiltration of white cells as well as a considerable amount of necrosis when it has been present in the host for 6 days (see also 2).

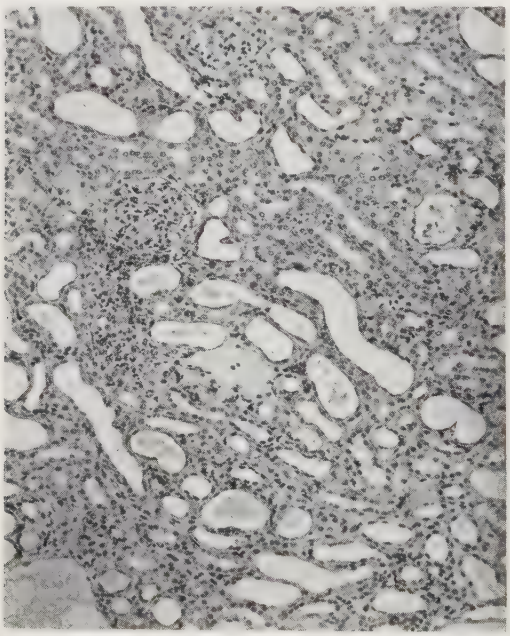

FIG. 2. Section from kidney transplant 6 days after introduction into host (H & E, 100×). This kidney was transplanted without an interruption in its blood supply. Note lack of white cell infiltration.

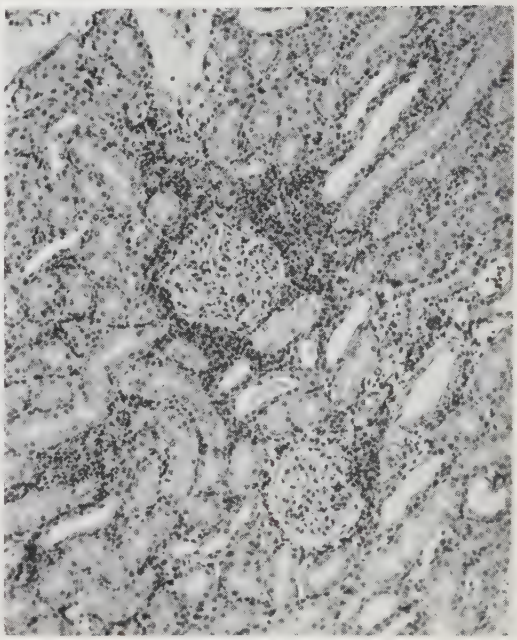

FIG. 3. Section from kidney transplant showing a marked infiltration of white cells, 6 days after introduction into the host (H & E, 160×). This kidney was subjected to a period of ischemia during the operation.

Summary. A technic is described for transplantation of the dog kidney from one animal to another without any interruption of the organ's blood supply. An accessory circulation from the neck vessels of the donor animal is provided for the kidney during the time required to connect the renal vessels to the host's vascular system. This procedure permits the study of the transplant rejection process without the ischemia which is a complicating factor in the technics usually employed. Transfer of the kidneys without ischemia does not markedly prolong the period of function, however the pathological changes which occur within the first few days following transplantation may be delayed.

After this work was completed and the manuscript submitted for publication, our attention was drawn to the papers of Paul Govaerts who used similar but not identical procedures to those described

in our article to carry out acute experiments on the effect of diuretics on kidney function (P. Govaerts, *C. R. de la Soc. Biol.*, 1928, v99, 647; *Arch. Internat. Pharmacodyn. et de Thérapie*, 1929, v36, 99). The technic was also employed by Brull (*C. R. de la Soc. de Biol.*, 1931, v107, 248, 249) for short-term experiments.

1. Williamson, C. S., *J. Urol.*, 1923, v10, 275.

2. Simonsen, M., *Biological Incompatibility in Kidney Transplantation in Dogs*, Ejnar Munksgaard, Copenhagen, 1953.

Received June 27, 1957. P.S.E.B.M., 1957, v96.

Colorimetric Method for Estimating Number of Cells in Monolayer Cultures without Physiological Damage.* (23463)

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A colorimetric method for determining the number of cells in monolayer cultures of monkey kidney cells has been developed. The method depends on production of hydrogen ions by the cells and consists in measuring change in absorption of a standard physiological salt solution of phenol red after a 15-second exposure to the cultures. The procedure avoids the physiological damage usually resulting when cells are removed from the glass surface for counting and makes it possible to estimate reliably the number of cells in the particular culture which is being studied.

Studies on many thousands of cultures during the last 6 months have shown that the amount of phenol red acidified under these conditions is correlated with the number of cells in the culture over a wide range of cultivation conditions and of age. The method proved useful particularly as a method of selecting from among a large number of cultures those having the same cell number within ± 10%. The mechanism of the reaction is not completely understood, and it is therefore not possible to evaluate all of the factors which might give anomalous results. In this paper the details of the procedure and factors controlling the sensitivity of the method will be given. Some of the possible sources of error, as determined from these limited studies, will be considered.

* Aided by grant from The National Foundation for Infantile Paralysis.

Material and methods. Monolayer cultures of monkey kidney cells were grown out from freshly trypsinized rhesus monkey kidneys in either medium M + H (0.5% lactalbumin-hydrolysate, 2% calf serum, Hanks' salt solution) (1) or the completely synthetic, protein-free medium SM-2(2) in 2-3 oz Brockway prescription bottles. *Phenol red* was the commercial LaMotte soluble product used either as obtained or after recrystallization from dilute alcohol. *Cell counts* were done by a modification of the Parker(3) method for counting nuclei. The modifications consist in using small volumes of citric acid to obviate the need to concentrate the cells by centrifugation and in adding the crystal violet within at least 5 minutes after the citric acid in order to stop the continued swelling and bursting of the nuclei. The exact procedure is as follows: 1) The supernatant fluids from the cultures are decanted, the bottles drained, and the cell layer covered with 1% tannic acid for 1½-2 minutes. 2) The tannic acid is decanted, the bottles drained on adsorbent paper, and exactly 2 ml of 10% citric acid is added to the cell layer. 3) After about a minute in contact with the citric acid, the bottles are shaken vigorously for 10-15 seconds. The time required to remove the cells from the glass and the amount of shaking depends on the age of the cells and whether they have been grown in a serum-containing or protein-free medium. With both cell types the older cultures (more than 5 days) are removed easily in 15-30 seconds, whereas younger cul-

tures may take as much as 5 minutes with vigorous shaking. Three- to 5-day-old cultures grown in synthetic medium may take as much as 5 minutes with vigorous shaking. 4) The nuclei are stained by adding 0.5 ml of 0.1% crystal violet in 0.1 M citric acid (4). *Chemical determinations.* Total endogenous polysaccharide was determined with the anthrone reagent as modified by Fales(5). The monolayers were first collected by solubilization in 0.2 N NaOH for a minute. *Spectrophotometric determinations* were made using the Beckmann Model DU spectrophotometer. *Temperature* in all of these experiments was the room temperature of 24-25°C.

Preliminary studies. Physiological salt solution. These studies were done using a physiological salt solution similar to both Hanks and Earles, containing NaCl 8.0 g, KCl 0.4 g, $MgSO_4 \cdot 7\ H_2O$ 0.2, $CaCl_2$ 0.14, and Na_2HPO_4 0.426 g, per liter of demineralized distilled water. The combined solution has a variable pH which declines on storage, due to the slow supersaturation with CO_2. To be assured of the same pH, it is necessary to make up the medium as follows: The NaCl, KCl, and $MgSO_4$ are made at 10 times concentrated combined stock. The $CaCl_2$ is kept at a separate 10% solution. A quantitative 0.30 M solution of Na_2HPO_4 made from the commercial anhydrous form which is kept in a desiccator is made and distributed to test tubes which are tightly stoppered. Each time the physiological salt solution is constituted, a previously unopened tube of dibasic phosphate is used. The final solution combined from the stocks diluted with distilled demineralized water equilibrated at room temperature (24-25°C) has a pH of 8.05 ± .05 as measured by the Beckmann Model H-2 pH meter. *Phenol red* is a weak acid which dissociates in the pH range 6.8 to 8.2. The peak of maximum absorption in the visible range of the spectrum in the physiological salt solution at alkaline pH is at 560-562 mμ. On acidification, the absorption at this region declines, with the appearance of an absorption band between 400 and 460 mμ. The absorption at 484 mμ is invariant with pH. It has been found that phenol red obeys Beers' law within the range of concentrations which can

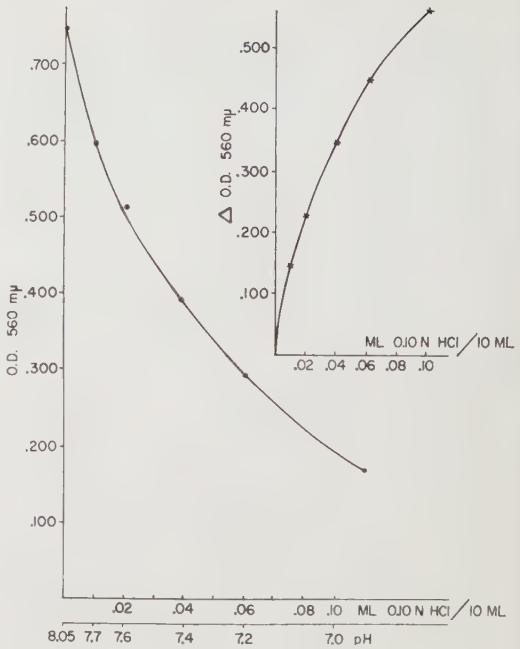

FIG. 1. Absorption of standard phenol red solution at 560 mμ between pH 8.0 and 7.0.

FIG. 1a. Change in absorption (delta o.d.) of standard phenol red solution as a function of amount of acid (△ H^+) added to 10 ml of the salt solution.

be measured directly. It has also been found that when working at the level of 6-8 g per liter of NaCl, as in physiological salt solutions, the extinction is not affected by changes in the concentration of the common ions.

The change in adsorption at 560 mμ on addition of measured quantities of 0.10 N HCl is shown in Fig. 1. It is clear that the change in absorption (delta o.d.) for a given quantity of acid (delta H^+) is constantly declining in the pH range of 8-7. This relationship is seen more clearly in Fig. 1a where the delta o.d. has been plotted against delta H^+. These data mean that when phenol red is used to measure a change in H^+, the change in absorption must be calibrated by titration with a standard acid, in order to eliminate differences in absolute quantities of phenol red, the amount of true titratable phenol red in the preparation, and to a lesser extent the sensitivity of the spectrophotometer used to measure delta o.d. Since the absorption peak at 560 mμ is very sharp, the band width of the light incident on the sample will influence

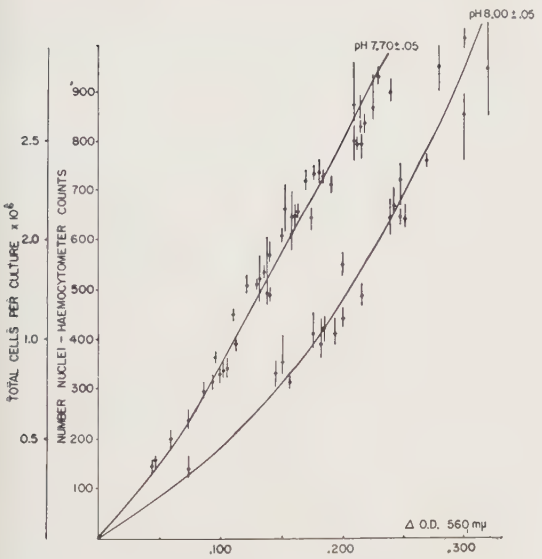

FIG. 2. Relationship between phenol red index and cell number in monolayer cultures of monkey kidney cells. Mean avg nuclei count plotted against change in absorption (△ o.d.) at 560 mμ during a 15-sec. exposure of the cultures to standard phenol red solution containing 3×10^{-3} M Na_2HPO_4 at pH 8.05 or adjusted to pH 7.70 with standard acid. Total fluctuation in nuclei counts (3-4 samples) indicated by bars.

the magnitude of the delta o.d. with the same delta H^+.

Procedure to determine cell number. The method depends on determining amount of hydrogen ion produced by monkey kidney cells, by determining the change in absorption at 560 mμ of a standard phenol red solution. The procedure is as follows: The medium from the culture to be titrated is decanted and the bottles drained 10-15 seconds. The residual culture medium is removed by rinsing the bottles 2 times with 10-15 ml portions of the physiological salt solution and the bottles drained. The bottle is placed so that the standard phenol red solution can be pipetted directly onto the cell layer. Using a stop watch timed from the second that the solution touches the cell layer, 3.0 ml of the standard solution is added to the bottle. After 15 seconds, the fluid is decanted and absorption at 560 mμ is determined. The difference in absorption between the standard solution and the effluent solution (phenol red index) is determined.

The phenol red index was determined on more than a hundred monolayer cultures of monkey kidney cells and compared with cell number as determined by counting nuclei of the same cultures. The cultures had been grown out in 2 different media, a serum containing medium, and the completely synthetic medium SM-2, and were studied between the 3rd and 35th day of *in vitro* life. Several cultures which had degenerated because the medium had not been changed were intentionally included. The data for these calibration curves have been presented in Fig. 2. It is seen that the change in absorption is correlated within ± 10% with the number of cells (nuclei) in the cultures.

The pH of the effluent solution as determined by the Beckmann Model H-2 pH meter and the pH expected from the change in o.d. (Fig. 1) agreed within the limits of measurement with this meter (± 0.1 pH unit). The change in absorption therefore can be ascribed primarily to production of hydrogen ions. The calibration curves show that the sensitivity of the method is greater with the pH of the standard solution at 8.0 than at 7.7. This agrees with the titration curve of the phenol red solution (Fig. 1a). The calibration curves also show that the delta o.d. is not a straight line function of cell number, but as would be expected from Fig. 1a, decreases with increasing cell number. Since the method is actually measuring delta H^+ as a result of cell number, it is useful to plot delta H^+ as a function of cell number. This may be done (Fig. 3) by determining the delta H^+ from the delta o.d. (Fig. 1a) for a given cell number (Fig. 2). It is seen that the amount of hydrogen ion produced is a linear function of cell number. This finding indicates that between pH 8.0 and pH 7.2, the range indicated by maximum delta o.d., the mechanisms involved in the production of hydrogen ion by the cells are independent of pH of the medium.

The phenol red index is now determined routinely on all cultures grown out from the trypsinization fluids in order to select cultures having the same cell number within a desired range for further study. The manipulations involved, including timing of the rinses,

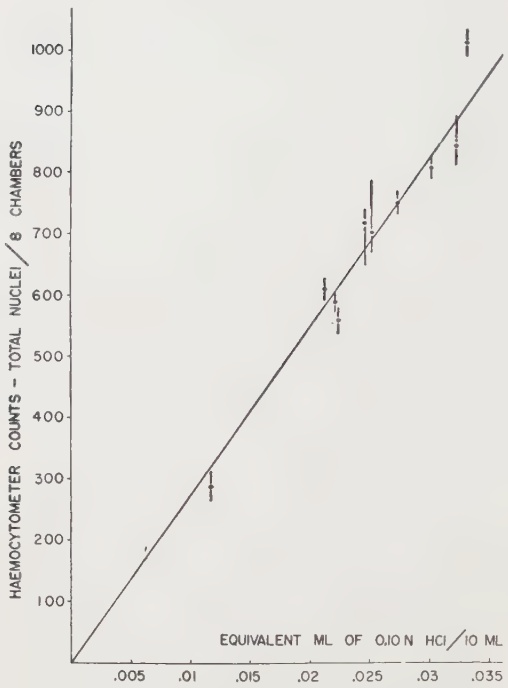

FIG. 3. Amount of H^+ produced as a function of number of cells in monolayer monkey kidney cultures. pH of the standard solution was varied between pH 7.4 and 8.0 ± .05.

amount of fluid used to rinse or pH of the rinse solution between 5.7 and 8.0 have not been found to affect the phenol red index. One obvious difficulty when large numbers of cultures are studied is the change in pH of the standard phenol red due to diffusion of CO_2 from the air. This may be obviated simply by covering the solution with mineral oil (Dracol) and using a dispensing flask with an outlet at the bottom attached to an automatic Cornwall pipetting outfit. The connection to the pipetting outfit should be of glass except for the minimum amount of gum rubber needed for manipulation. In experiments where a large number of bottles are being screened, time of exposure has been reduced to 10 seconds, which is within the time error of manipulations when using the automatic pipetting outfit. When screening a large number of cultures it was also useful to use a spectrophotometer not requiring special cuvettes. We have used both the Bausch and Lomb Spectronic 20, and the Beckmann Model C with a Corning glass 560 mμ filter and matched ordinary test tubes. With these instruments the accuracy is somewhat less than with the Beckmann model DU and is about ± 15%.

Standardization of phenol red solution. It has been shown that the amount of hydrogen ion produced by monolayer cultures of monkey kidney cells is a function of the number of cells in the culture. The amount of hydrogen ion may be determined, as was done above directly from the delta o.d. of the standard phenol red solutions, provided two criteria are met. 1) The standard solution must have the same pH, since the delta o.d. with delta H^+ varies with pH (Fig. 1). A method has already been given for obtaining a standard solution with a constant pH. 2) The standard solution must have a constant quantity of titratable phenol red. In the salt solution used, the buffering capacity of the medium is due to the Na_2HPO_4. Since phenol red obeys Beers' law, a 2-fold difference in concentration of phenol red gives a 2-fold increase in the delta o.d. for a given delta H^+. When using recrystallized phenol red solution, a constant quantity of phenol red may be obtained by determining the absorption at 560 mμ. Since most commercial preparations of phenol red are impure, and since in fact even recrystallized material changes on standing, the extinction at 560 mμ is not a sufficient criterion of the amount of titratable phenol red in the solution. One method for adding the same amount of titratable phenol red when using different commercial samples is as follows: From a 0.5% stock solution in water, 0.05 ml, 0.10 ml, and 0.150 ml are added to 100 ml aliquots of the salt solution. The o.d. at 560 mμ of these solutions is determined and the delta o.d. obtained on adding 0.01 ml of standard 0.1 N HCl per 10 ml for each of the solutions is determined. Delta o.d.'s are plotted versus milliliters of the phenol red added; the amount of stock solution giving a desired delta o.d. for the standard solution is extrapolated from the curve. For these studies, a delta o.d. of 0.150 ± .005 was selected. This represents a concentration of 5.0 mg per liter, with our best recrystallized phenol red preparation. Several commercial preparations have been standardized in this

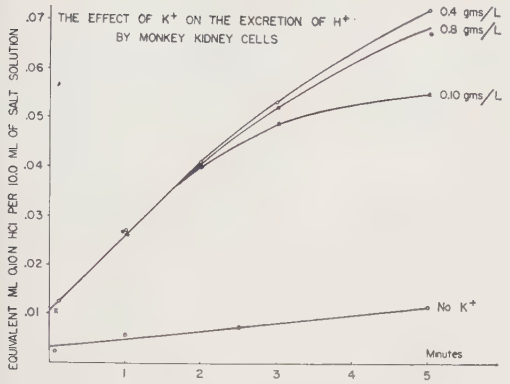

FIG. 4. Kinetics of H^+ production and requirement for K^+. Cultures had been selected from a series of 9-day-old "M" cultures to have the same phenol red index.

way and found to give the same phenol red index as the recrystallized material. The phenol red stock solutions are stable for several weeks if kept in the dark.

Kinetics of acidification of phenol red and effect of Na^+ and K^+. The amount of H^+ produced was determined as a function of time. It is seen (Fig. 4) that the rate is constant between 5 seconds and 2-3 minutes. Since this rate does not extrapolate back to zero, there is indication of a very rapid primary reaction. The K^+ has been found to be very critical for the H^+ production. As shown in Fig. 4, the production of H^+ is almost completely suppressed in the absence of K^+ in the salt solution. It is limited after 2 minutes at a concentration of 0.1 g/liter of K^+, even in the presence of high Na^+ concentration (8 g NaCl/liter).

In contrast, the effect of the Na^+ was not so great in this system. In Table I, changes in absorption and amount of acid produced in the salt solution without NaCl are given. The concentration of Na^+ could not be reduced below 0.134 g per liter, because of the presence of Na_2HPO_4 needed for the buffer. Since omission of NaCl significantly changes the spectrum of phenol red, it was necessary to retitrate the phenol red with standard acid in this solution. Table I shows that there was no significant effect on the primary reaction in the absence of NaCl; the secondary reaction was only slightly inhibited.

Invariance of the phenol red index with endogenous polysaccharide reserves. In the studies already presented, the production of hydrogen ions by monkey kidney cells was followed in a solution in the absence of any exogenous carbon. If energy is required for this reaction it is presumably made available by the endogenous polysaccharide reserves of the cells. Thus if the phenol red index was dependent on a critical level of these reserves it might give erroneous indication of cell number under different metabolic regimes. In an effort to check this the phenol red index was studied in cultures which had different levels of endogenous reserves.

The amount of polysaccharide (anthrone-positive material) in cultures of monkey kidney cells varies with age of the culture and with frequency of replenishment of the medium. The variations in the level of endogenous polysaccharide reserves in cultures between the 8th and 12th day after seeding and the effect of time of replenishment are shown in Table II. For this experiment the cultures had been matched for cell number on the 4th day after seeding by taking the phenol red index. At the time of the experiment the phenol red index was determined for each culture before the anthrone test. Several cultures of

TABLE I. Kinetics of H^+ Production in Salt Solution with Different Concentrations of NaCl.

0.134 g Na^+*			3.22 g Na^+		
Time	△ o.d.	Equivalent ml 0.1 N HCl/10 ml of same low sodium salt sol.	Time	△ o.d.	Equivalent ml 0.1 N HCl/10 ml of same salt
7 sec.	.115	.011	5 sec.	.155	.011
1 min.	.170	.019	1 min.	.265	.027
2 "	.210	.030	2 "	.415	.053
5 "	.300	.052	5 "	.470	.067

9-day-old cultures had been selected to have the same phenol red index.

* Contributed by the Na_2HPO_4 used for buffer. Salt solution had no added NaCl.

TABLE II. Changes in Endogenous Polysaccharide Reserves and Invariance of the Phenol Red Index.

Age of culture and replenishment schedule	Phenol red index (pH 8.0)	Equiv. cell No. $\times 10^6$ $\pm$ 10%	Cell No. $\times 10^6$ (avg haemocytometer count)	γ equivalents of glucose	
				Total per culture	Per 10^6 cells
8 days old—replenished at 6 days	.290	2.81		60	21
	.280		2.32		
	.272	2.5		49	21
	.273	2.5		51	20
	.280		2.40		
	.278	2.6		52	20
	.285	2.72		55	20
	.275	2.55		50	19
9 days old—replenished at 6 & 8 days	.320		3.31		
	.300	2.95		105	35
	.310	3.12		120	37
	.340	3.45		130	38
9 days old—replenished at 6 days	.265	2.40		32	13
	.270	2.50		28	11
	.270	2.50		30	12
	.260	2.35		27	11
	.280		2.52		
	.275	2.55		32	12
12 days old—replenished at 6, 8, 10 & 11 days	.275	2.50		180	72
	.285	2.72		205	75
	.280	2.60		192	74
	.282	2.60		187	72
	.277		2.55		

Cultures had been matched for cell number on the 4th day of outgrowth.

each series were also used for cell counts. The number of nuclei recovered agreed within $\pm$ 10% with the number expected from the phenol red index. On the other hand, on the basis of cell count the polysaccharide reserves varied between 11 γ and 70 γ per 10^6 cells. It may be concluded that the phenol red index is independent of endogenous polysaccharide reserves above a level of 10 γ per 10^6 cells.

The effect of severe starvation in a salt solution on the phenol red index has been studied. The phenol red index and the total nuclei were determined in a series of matched cultures which were changed from the M-H medium to Hanks salt solution containing no glucose. The number of cells expected from the phenol red index and the number of nuclei actually recovered after fixing with tannic acid and shaking with citric acid have been plotted at different times during the starvation. It is seen (Fig. 5) that when 7-day-old cultures are changed from the complete serum-containing medium to the salt solution without glucose, there is a rapid dissolution of the cells. This was reflected not only in decrease in the number of nuclei actually recovered but also in the uneven staining and broken membranes of those nuclei which were recovered. The count is only an approximate indication of the number of normal cells in the cultures. The count of cells expected from the phenol red index was significantly lower during the first 8 hours than the number of nuclei recovered. At 24 hours, however, the phenol red index was a reliable indication of the number of nuclei in the culture recoverable under these conditions. Rate of decline of the phenol red index during the first 8 hours is only about 2 times the rate of loss of recoverable nuclei. The observation suggests that mechanisms involved in excretion of hydrogen ions by the cells are maintained by the lowest metabolic rates compatible with the integrity of the cell.

Acid properties of glass. The excretion of H^+ shown to occur in salt solution presumably continues to some extent during growth. Since glass binds H^+ during rinses with strong acid, or even during autoclaving in water, it may be expected that the glass be-

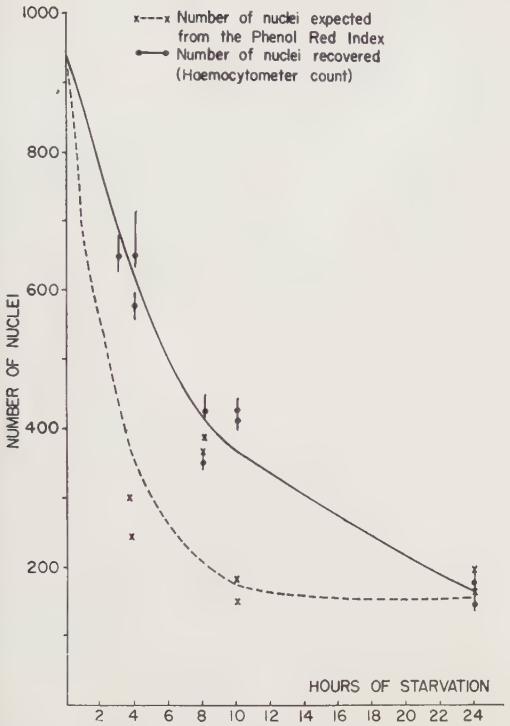

FIG. 5. Effect of starvation on phenol red index. Seven-day-old cultures transferred after rinsing in salt solution from the lactalbumin-hydrolysate-calf serum medium to Hanks salt solution without glucose. Number of nuclei indicated by value of the phenol red index determined at different times during starvation is compared to the number of nuclei recovered after treatment with citric acid.

comes acid during growth. That glass does retain H^+ is shown by the fact that the side of the glass on which the cells were not grown will acidify phenol red. Indeed, as will be discussed elsewhere, glass scraped clean of cells will still acidify phenol red. The glass used in these studies does not bind H^+ significantly before the cells have attained a population of 0.5-1.0 x 10^6, at which time the glass is nearly covered. If the phenol red solution is placed directly onto the cell layer, the contribution from the glass is negligible and has already been accounted for in the calibration curve. Glass, however, differs in the rate at which it binds H^+. It is probable that the calibration curve is valid only for cultures growing in the same type of glass.

Discussion. It has been found empirically that the amount of hydrogen ions excreted by monolayer cultures of monkey kidney cells is correlated with number of cells in the culture over a wide range of cultivation conditions and of age. The amount of hydrogen ions excreted seems to be independent of levels of endogenous polysaccharide reserves of the cells. Even under conditions of extreme physiological stress, *i.e.,* starvation in a salt solution without glucose, rate of hydrogen production decreased at only twice the rate of the decline in cell number. The results suggest that the excretion of hydrogen ions is a primary function of the cell which is maintained by the lowest metabolic rates compatible with the integrity of the cell.

The excretion of hydrogen ions has been studied extensively in perfused kidney(6), in stomach mucosa(7), and in yeast(8). Preliminary studies in this laboratory indicate that it is a characteristic function of other mammalian cells growing *in vitro.* The mechanism of hydrogen ion excretion is not understood and the studies presented here were designed only to evaluate the possible sources of error when it is used as a quantitative indication of cell number. Unfortunately, all the possible errors can not be evaluated until the mechanism has been clarified. It would seem, however, that mammalian cells growing *in vitro* may be used for quantitative studies on the mechanism of ion exchange.

Summary. A method has been presented for determining the number of cells in monolayer cultures of monkey kidney cells without physiologically damaging them. The method consists of determining the change in absorption of a phenol red solution after a 15-second exposure of the cultures under standard conditions. These conditions include the use of a standard solution defined with respect to pH, quantity of titratable phenol red, and buffering salt, and the use of the same type of glass in the different cultures. The change in absorption appears to be a simple consequence of the production of hydrogen ion by the cells. It is critically dependent on the presence of potassium but independent of an exogenous carbon source, and, independent of endogenous polysaccharide reserves over a wide range. The amount of hydrogen ion produced may be sufficiently independent of cell age

and metabolism to be a reliable method for the estimation of growth in cultures.

1. Melnick, J. L., and Riordan, J. T., PROC. SOC. EXP. BIOL. AND MED., 1952, v81, 208.

2. Rappaport, C., and Melnick, J. L., *Fed. Proc.*, 1957, v16, 429; Melnick, J. L., Hsiung, G.-D., Rappaport, C., Howes, D., and Reissig, M., *Texas Rep. Biol. and Med.*, 1957, in press.

3. Parker, R. C., Healey, G. M., and Fisher, D. C., *Canad. J. Biochem. and Physiol.*, 1954, v32, 306.

4. Sanford, K. K., Earle, W. K., Evans, V. I., Waltz, N. K., and Shannon, J. E., *J. Nat. Cancer Inst.*, 1951, v11, 773.

5. Fales, F. W., *J. Biol. Chem.*, 1953, v113, 193.

6. Pitts, R. F., and Alexander, R. S., *Am. J. Phys.*, 1945, v144, 239.

7. Davenport, H. W., *Phys. Rev.*, 1946, v26, 560.

8. Conway, E. J., and Brady, T., *Nature*, 1946, v159, 137.

Received July 3, 1957. P.S.E.B.M., 1957, v96.

Diabetes Mellitus and Serum Vitamin B_{12} Concentrations: 333 Patients. (23464)

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Since a role for vit. B_{12} in transmethylation and the transformation of carbohydrate to fat has been invoked(1), it is natural that deviations in the B_{12} metabolic pattern should be looked for in the most obvious defect of carbohydrate metabolism, human diabetes mellitus. It is conceded that patients with diabetes show an accelerated rate of development of cardiovascular degenerative disease, not directly correlated with age of onset, duration, or severity of the diabetes. Vascular disease of the retina, diabetic retinopathy and glomerulosclerosis with or without the Kimmelstiel-Wilson syndrome, are regarded as advanced manifestations of vascular degeneration. Observations correlated with these conditions raise the question whether the relationship is with severe vascular disease independent of diabetes or with diabetes itself.

Methods. Patients from 4 large diabetic clinics* were studied. Single serum samples were obtained from fasting patients and were assayed for their B_{12} content by the *Lactobacillus leichmanii* method(2,3,4). All sera were frozen and held at –20°C until assay and those specimens which were *not frozen* upon receipt were discarded. The diagnosis of diabetic retinitis in the patients studied was accepted only on the basis of an examination by an experienced ophthalmologist within a week of assay. The diagnosis of diabetes in all patients studied was established by history and the requirement of insulin for prolonged periods.

Results. The results are recorded in Tables I to IV. Normal groups for comparison were not obtainable from all institutions for it is not common for normal individuals in any number to be institutionalized. The statistical methods seemingly most appropriate to this study have been employed in the analysis of the data(5,6). Certain variables inherent in such a study as has been carried out, cannot be eliminated. The onset of diabetes mellitus is superimposed upon *pre-existent* physiology and pathology and diabetes in "pure" form in a patient free from abnormalities which, in the absence of diabetes would be regarded as falling within the limits of normal, is rare.

It is clear that where controls were available there was no consistent difference between diabetics and these "normals." Similarly, in the 3 studies where the subjects had been screened for retinopathy there was no apparent difference between patients with and

* The authors wish to express their indebtedness to Dr. Wallace W. Dyer of the Philadelphia General Hospital and Dr. Harry S. Lipscomb of Jefferson Davis Hospital, Houston, Texas, for their kindness in having supplied us with the sera from diabetic patients.

TABLE I. B_{12} Levels ($\mu\mu$g/ml) in Diabetics (PaH).

	♂		♀	
	No retinopathy	Retinopathy	No retinopathy	Retinopathy
Avg B_{12}	640	520	970	980
Range B_{12}	380–970	260–780	580–1440	260–2490
Avg age	68	63	65	57
No. subjects	5	2	4	12

There was no obvious difference between subjects with retinopathy and those without retinopathy. The females had significantly ($P < 0.05$) higher levels than the males. No "normal" controls were available for this study.

without retinal disease. The smallness of numbers raises some question of significance but the numbers present a true reflection of occurrence of retinopathy in the groups studied. There was some suggestion that the female diabetics have slightly higher levels than the males; this did not seem to be true for the normals included as controls. In other studies(3,4) there has been a suggestion that normal females have slightly higher, but not significantly different, levels than males.

Discussion. As pointed out, the question can be raised about the appropriateness of interpreting changes in vit. B_{12} metabolism observed in the presence of diabetic retinitis and glomerulosclerosis as correlating with diabetes rather than with severe vascular damage from any cause. Serum levels have been reported to be higher in diabetics in the presence of diabetic retinitis than in its absence(7). On the other hand, lower concentrations in the presence of severe retinopathy and glomerulosclerosis than in uncomplicated diabetes have been demonstrated(8). It has been implied, rather than established, that retinal vascular damage directly reflects similar systemic vascular damage. This may be so, but not necessarily, as has been pointed out in an excellent pathologic survey that states, ". . . retinal arteriosclerosis occurred more frequently in the absence of than with glomerular arteriosclerosis"(9).

Similar disagreement of observations is recorded with regard to the urinary excretion

TABLE II. B_{12} Serum Levels ($\mu\mu$g/ml) in Diabetics (NSH).

	♂			♀		
	No retinopathy	Retinopathy	Normal	No retinopathy	Retinopathy	Normal
Avg B_{12}	610	310	580	860*	760	570
Range B_{12}	250–870	—	100–1620	400–3400	1520–1020	20–2170
Avg age	49	52	42	60	53	59
No. subjects	6	1	108	14	6	238

* If one high value is omitted, the avg becomes 0.66.

There were too few values to make a valid test for effect of retinopathy. Avg level was higher for females than for males, but difference was not significant statistically ($P = 0.20$).

Avg B_{12} blood level was essentially the same for males and females in the "normal" controls. If the one high value in the diabetic group is omitted, the difference between diabetics and "normal" controls is not significant statistically ($P > 0.10$).

TABLE III. B_{12} Serum Levels ($\mu\mu$g/ml) in Diabetics (PGH).

	♂		♀	
	No retinopathy	Retinopathy	No retinopathy	Retinopathy
Avg B_{12}	480	480	600	630
Range B_{12}	80–1470	220–1010	140–1260	150–1230
Avg age	58	67	54	61
No. subjects	47	5	62	19

There was no obvious difference between subjects with retinopathy and those without retinopathy. The females had significantly ($P = 0.01$) higher levels than the males. No "normal" controls were available for this study.

TABLE IV. B_{12} Blood Levels ($\mu\mu$g/ml) in Diabetics (JDH).

	♂		♀	
	Diabetic	Normal	Diabetic	Normal
Avg B_{12}	650	660	780	780
Range B_{12}	80–1560	260–2100	70–1890	260–3000
Avg age	55	41	56	39
No. subjects	42	40	108	34

The diabetic females showed a tendency for higher levels than the males, but the difference did not reach the customary 5% level of significance ($P = .09$). The subjects were not screened for retinopathy.

In "normal" controls, the apparent sex difference was not significant statistically ($P = 0.21$). There was no obvious difference between diabetics and "normal" controls.

of vit. B_{12}; some investigators state that excretion of vit. B_{12} is *greater* in diabetics with retinopathy than in those without, and *higher* in diabetics than in normals(10). Others have found that there were no differences between such groups of patients(11). This investigation was directed toward determining in a large group of diabetics whether or not there was any difference in the serum B_{12} concentrations of such persons and non-diabetics. Normal values of serum vit. B_{12} have been previously established(3) as averaging 560 $\mu\mu$g/ml (70-1060 $\mu\mu$g/ml). This normal figure conforms with other published work (12) and statistically significant deviations from such normal values have been established in hepatitis(13,14), acute(15) and chronic myologenous leukemia(16,17), in pregnancy(18) and in newborns(4,19). Our study has shown (a) no difference between diabetics and normals, (b) a slight difference between male and female diabetics in favor of the females, and (c) no difference between patients with and without diabetic retinopathy. In face of these observations it is suggested that abnormal serum concentrations of B_{12} or alterations of urinary excretion of this vitamin are more probably related to extensive vascular damage *per se,* than to the metabolic disease, diabetes. The liver is known to be the chief reservoir for vit. B_{12} and the transport of the vitamin in the body has been related to blood proteins. That abnormal liver function can influence serum B_{12} concentrations has been shown(13,14). Since the liver is not uncommonly involved in diabetes, hepatic function may be responsible for some abnormal serum concentrations observed in diabetes.

There have been suggestions that the glucose tolerance test can be altered by B_{12}(20) and that the insulin requirement of diabetics can be altered by administration of the vitamin(21,22). Favorable and unfavorable reports of the benefit of B_{12} therapy for neuropathy in the presence of diabetes have been published. Certainly there has not been the uniformity of response that has been observed in the neuropathic manifestations of vit. B_{12} deficiency states(23,24).

Conclusion. A study of serum vit. B_{12} concentrations has failed to show significant differences between 333 diabetics and 420 normal persons, between 45 diabetics *with* and 138 *without* retinopathy, and only a slight difference between 225 females and 108 males in favor of the females. Within the limits of this study, the serum B_{12} values of diabetic subjects are the same as those for normal individuals. It is suggested that previously observed differences may reflect vascular damage rather than the diabetic state *per se.*

1. Ling, Chiun T., and Chow, Bacon F., *J. Biol. Chem.,* 1954, v206, 797.

2. Skeggs, H. R., Nepple, H. M., Valentek, K. A., Huff, J. W., and Wright, L. W., *ibid.,* 1950, v184, 211.

3. Boger, William P., Wright, Lemuel D., Strickland, S. Clyde, Gylfe, Julina S., and Ciminera, Joseph L., PROC. SOC. EXP. BIOL. AND MED., 1955, v89, 375.

4. Bayne, Gilbert M., Wright, Lemuel D., Beck, and George, S., *N. Eng. J. Med.,* 1957, v256, 1085.

5. Rosenbaum, S., *Ann. Math. Stat.,* 1954, v25, 146.

6. Kruskal, William H., and Wallis, W. Allen, *J. Am. Stat. Assn.,* 1952, v47, 583.

7. Chow, Bacon F., Rosen, David A., and Lang, Calvin A., PROC. SOC. EXP. BIOL. AND MED., 1954, v87, 38.

8. Sauer, H., and Heinrich, H. C., Vit. B_{12} Studies in Diabetes Mellitus: Vit. B_{12} and Intrinsic Factor, Hamburg, Germany; May 23-26, 1956.

9. Alpers, Bernard, J., Forster, Francis M., and Herbut, Peter A., *Arch. Neurol. and Psych.,* 1948, v60, 440.

10. Becker, Bernard, Langu, Calvin A., and Chow, Bacon F., *J. Clin. Nutr.,* 1953, v1, 417.

11. Bookman, John J., Joelson, Robert H., Toll, William G., Baker, Herman and Dolger, Henry, *ibid.*, 1957, v5, 26.

12. Mollin, D. L., and Ross, G. I. M., *The Pathophysiology of Vitamin B_{12} Deficiency in the Megaloblastic Anaemias, Vit. B_{12} and Intrinsic Factor,* 414-430, F. Enke, Stuttgart; 1957.

13. Rachmilewitz, M., Aronovitch, J., and Grossowicz, N., *J. Lab. and Clin. Med.,* 1956, v48, 339.

14. Stein, Y., Stein, Olga, Aronovitch, J., Grossowicz, N., and Rachmilewitz, M., *Bull. Research Council,* 1956, v6E, 5.

15. Beard, Marion F., Pitney, W. R., Sanneman, Everett, H., Sakal, Jarvin J., and Moorehead, Harry H., *Ann. Int. Med.,* 1954, v41, 323.

16. Beard, Marion F., Pitney, W. R., and Sanneman, E. H., *J. Hemat.,* 1954, v9, 789.

17. Erdman-Oehlecker, S., *Der Vitamin B_{12} Stoffwechsel bei Hämblastosen: Vit. B_{12} and Intrinsic Factor,* 456-472, F. Enke, Stuttgart; 1957.

18. Boger, William P., Wright, Lemuel D., Beck, George D., and Bayne, Gilbert M., PROC. SOC. EXP. BIOL. AND MED., 1956, v92, 140.

19. Wright, Lemuel D., Bayne, Gilbert M., *Serum Vitamin B_{12} Concentrations of Pregnant Women and Infants, Vit. B_{12} and Intrinsic Factor,* 443-449, F. Enke, Stuttgart, 1957.

20. Malizia, E. F. del Greco, *Policlinico* (Sez prat.) 1951, v58, 1413.

21. Ralli, Elaine P., Barbosa, F. Xavier, Dumm, Mary E., Beck, Edith M., and Laken, Bertram, *J. Clin. Endocrinol.,* 1955, v15, 898.

22. Meites, Joseph, and Jeng, Y. S. L., *Fed. Proc,* 1955, v14, 100.

23. Sancetta, S. M., Ayres, P. R., and Scott, R. W., *Ann. Int. Med.,* 1951, v35, 1028.

24. Beidleman, B., *J. Clin. Nutr.,* 1953, v1, 119.

Received July 8, 1957. P.S.E.B.M., 1957, v96.

Acetoacetate Induced Dehydroascorbic Acid Accumulation in Blood and Tissues and Its Prevention by Glucose-Cyclo-Acetoacetate. (23465)

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Acetoacetate, an intermediary metabolite of lipids found by Nath *et al.*(1,2) to cause an immediate rise in blood glucose and decrease in glucose tolerance, has been reported(3) to cause a sudden increase in vit. C of blood of rats, when doses are relatively small. The higher doses (500 mg/kg or above) were, however, reported to depress such biosynthesis. Acetoacetate has also been reported to deplete GSH of blood of rabbits(4), and administration of the condensation product (Na salt) of glucose and acetoacetate (glucose-cyclo-acetoacetate) prior to injections of acetoacetate has been found to prevent this depletion of GSH and the rise in blood sugar(5). This depletion of GSH has been attributed to the oxidizing action of dehydroascorbic acid formed as a result of acetoacetate injections. The hydrolysed condensation product has been found by the authors(6) to act like GSH in converting dehydroascorbic acid back to ascorbic acid. It can be assumed that glucose-cyclo-acetoacetate exerts its sparing effect on GSH by undergoing enzymatic hydrolysis in the system. The present paper deals with the effect of glucose-cyclo-acetoacetate on accumulation of dehydroascorbic acid in blood and tissues and its excretion in urine as a result of acetoacetate injection in high doses.

Methods. Twenty-four male albino rats, of about 200 g each were divided into 4 groups, A, B, C, and D, of 6 rats each. Group A served as controls while Group B was given daily injection of Na-acetoacetate 500 mg/kg body weight. Group C were given daily 900 mg/kg body weight of condensation product (Na salt) prior to injections of 500 mg/kg of acetoacetate. In Group D, 900 mg/kg of hydrolysed condensation product (hydrolysed by 2N HCl for 10 minutes in boiling water, cooled and neutralized with NaOH to pH 7.2) was administered prior to acetoacetate injections daily. All injections were given intraperitoneally. The 24 hr urine of

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TABLE I.* Average Urinary Excretion of Ascorbic Acid (ASA) (mg) and Dehydroascorbic Acid (DHA) per Rat per 24 Hr on:

	1st day		3rd day		7th day		12th day		19th day		24th day	
Group	ASA	DHA	ASA	DHA	ASA	DHA	ASA	DHA	ASA	DHA	ASA	DHA
A	.85	.05	1.1	.09	1.15		.83	.04	1.05	.05	.95	.04
B	.53	.46	.44	.48	.38	.36	.31	.32	.36	.38	.26	.44
C	.74	.30	.88	.23	.92	.24	.78	.15	.94	.11	.88	.10
D	.92	.26	.95	.26	.92	.22	1.2	.91	.86	.26	.92	.22

* The groups are described in the text.

animals of different groups was collected in flasks containing 10 ml of metaphosphoric acid solution and the urine of each group of animals was pooled to find out the average volume of urine excreted per animal. Ascorbic acid was estimated by titrating against the standard 2:6 dichlorophenol indophenol solution and dehydroascorbic acid by passing H_2S gas and subsequent removal of excess of H_2S by CO_2 gas and titrating against the standard dye solution. Blood sugar was determined by the method of Hagedorn and Jensen(7). Rats from each group were sacrificed on the 7th, 13th, 19th and 24th day. Blood sugar, blood ascorbic and blood dehydroascorbic acids levels were determined. 10% trichloroacetic acid solution was used to deproteinize the blood and subsequent dilution was made with 6% trichloroacetic acid solution. The tissues (liver, kidney, spleen and adrenals) were macerated in sand with metaphosphoric acid-acetic acid mixtures for ascorbic and dehydroascorbic acid estimations.

Results. The 24 hour urinary excretion of ascorbic and dehydroascorbic acids of various groups estimated on different days of experiment is recorded in Table I. The levels of blood sugar and blood ascorbic and dehydroascorbic acids are summarized in Table II while the levels of the 2 acids in different tissues on 7th, 13th, 19th and 24th day have been given in Table III.

The animals given only acetoacetate (Group B) excrete more dehydroascorbic acid than the animals in other groups (Table I). Also the ascorbic acid excretion of that group is markedly less as compared to the other groups suggesting excess formation of dehydroascorbic acid. The blood sugar of Group B animals is also slightly higher than that in other groups. In the tissues of Group B animals, we find excess accumulation of dehydroascorbic acid, particularly in liver, spleen and adrenals as compared with other groups.

In Group C there is comparatively less dehydroascorbic acid and this comparison is well marked in spleen and adrenals though the livers of Group C animals do show some dehydroascorbic acid, though much less than in Group B. In the animals in Group D given hydrolysed condensation product before acetoacetate injection, dehydroascorbic acid is practically absent. The ascorbic acid contents also do not show any decrease.

Thus acetoacetate injections result in formation and accumulation of excess dehydroascorbic acid; GSH depletion following acetoacetate injections may be the result of its requirements for conversion of dehydroascorbic acid back to ascorbic acid.

Summary. 1. The effect of acetoacetate injections in doses of 500 mg/kg on ascorbic and dehydroascorbic acid contents of blood and tissues, and their excretion in urine, was

TABLE II.* Average Blood Sugar, Blood Ascorbic and Dehydroascorbic Acids on Days.

	Blood sugar, mg/100 cc blood				Ascorbic acid (reduced and oxidised) in mg/100 ml blood							
					7th		13th		19th		24th	
Group	7th	13th	19th	24th	ASA	DHA	ASA	DHA	ASA	DHA	ASA	DHA
A	102.8	98.8	105.1	95.5	1.85	nil	1.92	nil	1.97	nil	1.94	.04
B	114.6	123.4	131.3	138.8	1.24	.48	1.1	.62	1.15	.75	.96	.83
C	96.8	111.4	108.8	112.4	1.7	.30	1.8	.22	2.1	.21	2.0	.19
D	109.2	107.6	111.7	113.6	2.3	.16	2.2	.21	2.1	.22	2.1	.13

* The groups are described in the text.

TABLE III.* Reduced and Oxidised Ascorbic Acid (mg) per 100 g Tissue (Liver, Kidney, Spleen and Adrenal) at Various Intervals (Days).

Group	Liver		Kidney		Spleen		Adrenal		Liver		Kidney		Spleen		Adrenal	
	ASA	DHA	ASA	DHA	ASA	DHA	ASA	DHA	ASA	DHA	ASA	DHA	ASA	DHA	ASA	DHA
	7th day								13th day							
A	21.5	.21	17.5	nil	16.8	nil	267.4	1.3	20.8	.18	18.2	nil	17.1	.10	253.7	nil
B	16.9	9.5	12.8	3.2	13.3	7.9	175.5	114.8	13.1	10.3	10.1	3.3	12.4	11.6	204.2	138.8
C	22.9	3.2	19.9	2.8	17.2	1.6	320.3	17.8	18.8	5.3	19.8	2.3	19.1	.92	286.4	13.2
D	24.4	2.1	20.2	.40	15.6	1.4	328.9	nil	25.9	1.9	18.2	.24	16.1	nil	326.9	5.4
	19th day								24th day							
A	22.1	.12	17.4	.05	16.8	nil	299.8	.23	20.2	.21	18.1	nil	17.6	nil	336.8	nil
B	12.2	12.2	11.2	2.9	9.8	10.8	209.5	128.5	12.4	14.6	12.4	4.8	10.2	9.9	220.2	124.6
C	21.4	5.6	18.9	1.9	16.3	.71	340.4	8.9	20.7	4.2	20.3	2.1	17.1	.86	331.4	9.2
D	23.7	1.8	17.8	.32	16.1	nil	320.8	1.8	24.2	1.8	18.2	.21	15.9	.27	315.5	4.2

* Groups are described in the text.

studied. 2. Animals injected with acetoacetate excrete more dehydroascorbic acid and less ascorbic acid. Their tissues (liver, kidney, spleen and adrenal) also accumulate more dehydroascorbic acid, and the amount of ascorbic acid in them is diminished. 3. Glucose-cyclo-acetoacetate or its hydrolysed product has been found to prevent this increased accumulation of dehydroascorbic acid in blood and tissues. 4. Acetoacetate has been shown to cause oxidation of ascorbic acid to dehydroascorbic acid *in vitro* in presence of a few drops of $CuSO_4$ solution at a faster rate than that in the control. 5. The mechanism for prevention of acetoacetate-induced accumulation of dehydroascorbic acid by hydrolysed glucose-cyclo-acetoacetate has been suggested.

The authors are thankful to the Ministry of Education, Government of India for awarding the Government of India Senior Research Training Scholarship to one of them (R.M.B.).

1. Nath, M. C., and Brahmachari, H. D., *Nature,* 1944, v154, 487; *Ind. J. Med. Res.,* 1949, v37, 61.

2. Nath, M. C., and Chakrabarti, C. H., PROC. SOC. EXP. BIOL. AND MED., 1950, v75, 326.

3. Nath, M. C., Chakrabarti, C. H., Hatwalne, V. G., and Laul, K. S., *Nature,* 1948, v162, 660.

4. Nath, M. C., Hatwalne, V. G., and Gadgil, J. S., *Biochem. J.,* 1953, v53, 479.

5. Nath, M. C., and Behki, R. M., *Arch. Biochem. and Biophys.,* in press.

6. ———, *Metabolism,* 1957, v6, 127.

7. Hagedorn, H. C., and Jensen, B. N., *Biochem. Z.,* 1923, v135, 46.

Received July 8, 1957. P.S.E.B.M., 1957, v96.

Susceptibility of Pig Kidney Tissue Cultures to Certain Viruses. (23466)

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During attempts to replace the costly monkey kidney tissue cultures used for the diagnosis of various virus diseases, it was found that pig (porcine) kidney tissue cultures were susceptible to the Coxsackie viruses of Group B but were unaffected by the Echo virus group. A differential diagnostic test is thus suggested for these 2 groups of viruses. Although we could find no record of previous use of pig kidney tissue cultures for the propagation of viruses pathogenic for humans, the viruses of vesicular exanthema(1), vesicular stomatitis(2) and foot and mouth disease (2) have been shown to reproduce in such cultures.

Materials and methods. Kidneys were obtained either from suckling pigs, 3 to 6 weeks of age, in the laboratory or from adult pigs at an abattoir. In the latter case, kidneys were removed as aseptically as possible within 30 minutes of death of the animal, placed immediately into synthetic medium 150(3,4) to which 100 units/ml of penicillin, 100 μg/ml of streptomycin and 40 units/ml of mycostatin had been added. They were removed to the laboratory and immediately trypsinized at 4°C by the method of Bodian(5). For the initial cell growth period, 2% calf serum was added to the cells which were suspended in either Bodian's medium(5) or medium 150. Kidney cultures prepared from suckling pigs grew rapidly but had a shorter survival time than those prepared from adult pig kidneys. Since virus susceptibility was the same in both cases, adult pig kidneys were chosen for the work reported here. All of the established virus strains studied had been maintained in this laboratory in monkey kidney tissue cultures prior to the tests except the Coxsackie Types A1-A8 and A10-A19 which had been maintained by serial suckling mouse brain passage. Tissue cultures were observed for 7 days after inoculation at which time complete cell degeneration had taken place in those inoculated with cytopathogenic strains.

Results. A summary of the susceptibility of monkey kidney and pig kidney tissue cultures to the viruses studied is presented in Table I. The type of degeneration observed in pig kidney cultures was similar to that seen in monkey kidney cultures, that is, the appearance of round, refractile cells which finally fall away from the wall of the culture flask.

Echo virus Types 1-11 and 14-19, inclusive, have been studied in this work. With the exception of Type 10, which did cause cytopathic effects to some degree, these agents did not induce cytopathic changes in pig kidney tissue cultures.

Two to 4 serial passages were carried out with all strains and serum-virus neutralization tests with standard immune sera indicated that the specificity of the cytopathogenic strains had not been altered. Essentially the same virus titers were obtained with both monkey kidney and pig kidney infected culture fluids, varying from titers of 10^3 TCD_{50}

TABLE I. Cytopathogenicity of Various Viruses in Pig Kidney and Monkey Kidney Tissue Cultures.

Virus	Type or strain	Pig kidney	Monkey kidney
Coxsackie:			
Group B	B-1 to B-5 incl.	+	+
" A	A-1 to A-8; A-10 to A-19 incl.	—	—
	A-9	—	+
Echo*	Types 1 to 9; 11; 14 to 19 incl.	—	+
Poliomyelitis:			
Type I	Mahoney, Brunhilde	—	+
" II	MEF1, YSK, MEF1-mouse adapted	—	+
" III	Saukett, Leon	—	+
Virus B		+	+
Adenovirus†	Types 1 to 7 incl.	+	+

* Kindly supplied by Dr. Melnick's laboratory, New Haven, Conn., and Dr. A. B. Sabin's laboratory, Cincinnati, O.

† Kindly supplied by Dr. I. W. MacLean, Jr., Parke Davis Co., Detroit, Mich.

(tissue culture infective doses) for the adenoviruses to 10^7 TCD_{50} for the Coxsackie B viruses.

Several strains of Coxsackie virus Type B which had been isolated from human stool specimens in monkey kidney tissue cultures were also isolated in pig kidney cultures from the same specimens. On the other hand, poliomyelitis Types I, II and III viruses which had been isolated in monkey kidney cultures from similar specimens could not be isolated in pig kidney cultures.

Summary. 1) Susceptibility of pig kidney tissue cultures to the following viruses has been described: Coxsackie A and B, Echo, poliomyelitis, adenoviruses, and virus B. 2) Susceptibility of these cultures to Coxsackie group B viruses, virus B and the adenovirus group has been established. From the results it is apparent that pig kidney cultures may be utilized as an additional tool in the diagnostic differentiation between Coxsackie Group B viruses and the Echo virus group.

1. McClain, M. E., *et al.*, PROC. SOC. EXP. BIOL. AND MED., 1954, v86, 771.

2. Sellers, R. F., *Nature,* 1955, v176, 547.

3. Morgan, J. F., Campbell, M. E., and Morton, H. J., *Nat. Cancer Inst.,* 1955, v16, 557.

4. Morgan, J. F., Morton, H. J., and Parker, R. C., PROC. SOC. EXP. BIOL. AND MED., 1950, v73, 1.

5. Bodian, David, *Virology,* 1956, v4, 575.

Received July 10, 1957. P.S.E.B.M., 1957, v96.

Some Factors Affecting Differential Excretion of D-Phenylalanine in Man.* (23467)

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Marked variability between individuals in urinary excretion and utilization of D-phenylalanine by man has been noted by several investigators(1,2). It has also been recently observed through limited twin studies that some of this variability may have a genetic basis(2). Since the physiological basis of this differential excretion of D-phenylalanine could be quite complex, we felt it would be worth while to investigate various physiological aspects of this problem before going on with further genetic studies. Therefore, the present study of the role of the following physiological variables in the differential excretion of D-phenylalanine was undertaken: (1) absorption of D-phenylalanine into the blood, (2) metabolism of D-phenylalanine, and (3) renal excretion of D-phenylalanine.

Methods and materials. The subjects were normal, healthy adults and were tested after an overnight fast. At this time, blood and urine samples were obtained and the subjects were then given orally 0.5 g of D-phenylalanine (Mann Laboratories) dissolved in 200 ml of water. Blood was taken from the anticubital vein into heparinized tubes and the plasma drawn off. The plasma and urine samples were then stored at −10°C until ready for analysis. On 2 of the 4 occasions where experiments were repeated on the same individuals, 1.0 g of D-phenylalanine was given instead of 0.5 g for the second trial. One hour after feeding, blood samples were again taken and the urine flow for the one hour test period collected. L-phenylalanine determinations were carried out on all plasma and urine samples by a modification(3) of the Udenfriend and Cooper decarboxylase method(4). D-phenylalanine was estimated by the difference between L-phenylalanine determinations and paper chromatographic estimations of DL-phenylalanine on the second blood and urine samples. Phenylpyruvic acid determinations

* This work was supported by a grant from Natl. Science Fn.

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TABLE I. Summary of Data on Plasma and Urine Phenylalanine (Phe) and Urine Phenylpyruvic Acid (PPA) One Hour after Oral Administration of 0.5 g of D-phenylalanine.

Subject and sex	Age (yr)	Wt (kg)	Plasma, mg/100 ml: L-Phe: Fasting	L-Phe: Post feeding	Plasma: D-Phe	Urine (mg/hr): D-Phe	Urine (mg/hr): PPA	Renal clearance† D-Phe
1 ♀	20	54.4	1.4	1.3	1.7	59.7	15.4	58.6
2 ♀	20	65.8			.7	27.4	1.4	64.3
2 ♀*			1.0	1.3	1.6	98.6	8.5	102.5
3 ♀	30	63.5			.8	52.1	9.0	108.8
3 ♀*			1.3	1.4	2.4	99.0	24.4	68.3
4 ♀	19	54.9	1.3	1.2	.7	7.1	0	15.4
5 ♀	20	58.5			.9	23.4	2.1	43.3
6 ♂	33	80.3	1.1	1.0	1.2	18.2	.5	26.2
6 ♂						38.1	0	
7 ♂	34	63.5	1.4	1.4	1.0	35.7	11.2	59.5
7 ♂						57.9	7.0	
8 ♂	33	72.7	1.2	1.2	1.0	33.3	0	55.0
9 ♂	33	68.0	1.0	1.0	1.5	40.8	.5	46.1
10 ♂	46	65.5	1.2	1.3	1.2	37.4	9.3	52.5

* Fed 1.0 g D-phenylalanine.

† $\frac{\text{Urinary D-Phe (mg/min.)}}{\text{Plasma D-Phe (mg/ml)}}$.

were carried out on urine samples according to a method by Armstrong(5).

Results. In Table I are presented the pertinent data on the subjects tested and the results of the analyses of their blood and urine samples.

As can be seen, there is considerable variation between individuals in the urinary excretion of D-phenylalanine (the range being over 3 fold), and no apparent sex difference. In the case of individuals 6 and 7, experiments with the same test dose were repeated on separate occasions. Considerable variation exists between different trials on the same individual, but the relative and apparently absolute difference between these two subjects is constant. Clearly some of the apparent differences between individuals in the excretion of D-phenylalanine must be ascribed to individual daily variability. However, a considerable portion remains which may be due to individual physiological differences.

We shall consider first the possibility of differential absorption of D-phenylalanine into the blood as a possible physiological explanation of these individual differences. An estimate of this factor can be obtained from column 6 which gives the plasma level of D-phenylalanine one hour after the ingestion of the substrate. The range of variation is approximately 2-fold and there appears to be a general relationship between the plasma level of D-phenylalanine and the amount excreted in the urine. Statistical evaluation of this relationship yields a correlation ($\gamma = .57$) which is just significant at the 5% level. Thus differential rate of absorption of D-phenylalanine is of some importance in explaining the differential excretion of this substance.

We may next consider the possibility of the metabolism of ingested D-phenylalanine. There is considerable evidence(1,6) that D-phenylalanine may be utilized to some extent by man. Such utilization would require inversion of the D to the L form or oxidative deamination of D-phenylalanine to phenylpyruvic acid. These are not necessarily independent or mutually exclusive processes, since, in fact, inversion might require prior oxidation to phenylpyruvic acid.

In the fourth and fifth columns are given the fasting and the post-feeding plasma levels of L-phenylalanine. Any inversion of D to L should be indicated by an increase in the post-feeding plasma level of L-phenylalanine. Except for the second experiment on subject 2, there is no evidence for such conversion; in fact, the means of the 2 columns are equal. Therefore, if inversion of D to L is taking place in these experiments, the rate of inversion is so low that this process cannot be considered as contributing very much to the observed variation in D-phenylalanine excretion. However, in view of the evidence for

partial utilization of D-phenylalanine by man, it is most likely that inversion is occurring at a low rate in these experiments. Aside from experimental errors, there is an interesting point, peculiar to these experiments, which may explain our inability to detect inversion. This relates to the fact that D-phenylalanine through some inhibitive or competitive process increases the renal excretion of L-phenylalanine, thereby causing sufficient loss of plasma phenylalanine so that the effect of a slight amount of inversion (less than 5%) would be obscured. This phenomenon, together with further work, will be reported later.

In the next to last column is given the amount of phenylpyruvic acid excreted one hour after the feeding of D-phenylalanine. As can be seen, there is marked variation between individuals in this respect, with values ranging from less than 1 to over 15 mg of phenylpyruvic acid after the feeding of 0.5 g of D-phenylalanine. Considering this wide range of variation and the fair repeatability of experiments on the same individual, it would appear that this biochemical variation might be a profitable one for genetic studies. In this respect, it is interesting to point out that a similarly marked range of variation in the excretion of free phenols (presumably mainly p-hydroxyphenylpyruvic acid) following the feeding of D-tyrosine was observed by Albanese *et al.*(7). However, differences in the excretion of phenylpyruvic acid do not appear to account in any way for the observed variation in the excretion of D-phenylalanine. Small amounts of urinary phenylpyruvic acid would be associated with large amounts of urinary D-phenylalanine and vice versa, if variations in the excretion of D-phenylalanine were to be explained by differential excretion of phenylpyruvic acid. As can be seen, this is not the case, and furthermore, it is clear that the amounts of phenylpyruvic acid excreted relative to D-phenylalanine are small.

The final factor we should like to consider in the explanation of the variation of D-phenylalanine excretion is that of renal function. This could be an important factor if differential reabsorption of D-phenylalanine occurred in the tubules. To investigate this possibility, renal clearance values have been estimated and are given in the last column. Though these estimates of renal clearance were not obtained under rigorous physiological conditions, we feel that they do furnish a valid relative picture of the variation between individuals in the clearance of D-phenylalanine. As can be seen, there is a general correlation between the renal clearance values and the amount of D-phenylalanine excreted, the actual value of this correlation being .74, which is significant beyond the 5% level. Therefore, it would appear that differential tubular reabsorption of D-phenylalanine is an important determinant of the observed variation in the excretion of D-phenylalanine.

Discussion. From the data presented, it appears that differences in metabolism of D-phenylalanine are relatively unimportant variables in accounting for the observed variation in urinary excretion of D-phenylalanine. It seems, rather, that differences in absorption and renal excretion account for most of the observed variability in urinary excretion. In fact, considering that the latter two variables are independent (the correlation between absorption and renal excretion is not significantly different from zero), it appears that the combined variation in absorption and renal excretion can account for practically all of the observed variation in urinary excretion of D-phenylalanine under these experimental conditions.

However, in terms of genetic potentialities, it seems that excretion of phenylpyruvic acid after ingestion of D-phenylalanine is the most promising of the variables investigated. This is so because of the marked variation between individuals in excretion of phenylpyruvic acid and the repeatability of experiments on the same person.

Summary. This study represents an investigation into the physiological basis of the differential excretion of D-phenylalanine in man after its oral administration. Of the processes investigated: adsorption of the substrate into the blood, inversion to the L form, oxidative deamination to phenylpyruvic acid, and renal function, differences in absorption and renal function were important in accounting for the differential excretion of D-phenyl-

alanine.

We should like to acknowledge the assistance of Barbara Lenes Mohandes and Jayne Rosenthal of Barnard College.

1. Albanese, A. A., Irby, V., and Lein, M., *J. Biol. Chem.*, 1947, v170, 731.

2. Gartler, S. M., and Tashian, R. E., *Science*, 1957.

3. Tashian, R. E., and Gartler, S. M., *Amer. J. Hum. Genetics*,

4. Udenfriend, S., and Cooper, J. R., *J. Biol. Chem.*, 1953, v203, 953.

5. Armstrong, M. D., and Low, N. L., PROC. SOC. EXP. BIOL. AND MED., 1957, v94, 142.

6. Rose, W. C., Leach, B. E., Coon, M. J., and Lambert, G. F., *J. Biol. Chem.*, 1955, v213, 913.

7. Albanese, A. A., Irby, V., and Lein, M., *ibid.*, 1946, v166, 513.

Received July 10, 1957. P.S.E.B.M., 1957, v96.

Pyridoxal Phosphate (B_6-al-PO_4) Levels of Circulating Leukocytes in Maternal and Cord Blood.* (23468)

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In the later stages of pregnancy there exists a disturbance of Vit. B_6 metabolism. Following a tryptophan load test large amounts of xanthurenic acid appear in the urine(1) and the excretion pattern of tryptophan metabolites on chromatographic analysis deviates from the normal(2). This is immediately corrected by administration of relatively small amounts of pyridoxine(1,2). Furthermore pregnant women retain administered Vit. B_6 to a larger extent than do normal controls(3). The present report deals with the estimation of the coenzymatically available active form of Vit. B_6, namely pyridoxal phosphate, in the only readily accessible prototype tissue, *i.e.* leukocytes, in the blood of non-pregnant controls, mothers at term and in the cord blood of their babies.

Material and methods. Blood samples were taken from non-pregnant healthy women, women during delivery and from the cord blood of their babies. The pregnant women were divided into 3 groups. One group received no vitamin supplementation containing Vit. B_6. A second group had taken, during the course of their pregnancy, vitamin preparations which contained some Vit. B_6 in an uncontrolled and irregular manner. A third group had received, for a period of at least 14 days prior to their confinement, 2 tablets of 5 mg each pyridoxine hydrochloride daily. 5-10 ml venous blood was collected into siliconed test tubes containing a small amount of disodium versenate. Pyridoxal phosphate was estimated in isolated leukocytes by a recently developed method(4) which is based on the coenzyme function of B_6-PO_4 in a tyrosine decarboxylase system from *Streptococcus faecalis*(5). The leukocytes were isolated within a few hours after the blood had been obtained(5a). To the packed leukocytes 0.25 ml of 0.5 *N* NaOH was added and the tubes immersed in a boiling water bath for 5 minutes. The alkaline hydrolisates were kept in the refrigerator at 4°C and worked up within 24 hours. All analyses were made in triplicate and the samples were assayed at random.

Results. The results are summarized in Table I. In the first experimental group consisting of 60 non-pregnant, healthy women of child-bearing age, the amount of pyridoxal phosphate varied from 0.11 to 0.79 mγ/million leukocytes, averaging 0.32 ± 0.02 mγ/million leukocytes. The second group consisted of 51 women at term who had received no Vit. B_6 supplementation during their pregnancy. The B_6-al-PO_4 levels varied between

* This work was aided by grants-in-aid and material from Merck and Co. and National Vit. Fn. We are indebted for valuable advice to Dr. G. E. Boxer, Merck Inst. for Therapeutic Research.

TABLE I. Pyridoxal Phosphate (B_6-al-PO_4) Levels of Circulating Leucocytes.

Group	No. of cases	Age range	Avg age	Range of B_6-al-PO_4 content (mγ/million cells)	Avg B_6-al-PO_4 content (mγ/million cells)	Probability relation	
I. Non-pregnant controls (N)	60	19–45	28	.11– .79	.32 ± .02	M., N.V. *vs* N.	<.01
II. A. Pregnant women at term without vit. B_6 medication (M., N.V.)	51	19–40	27	.01– .36	.16 ± .01	M., N.V. *vs* C., N.V.	<.01
B. Cord blood (C., N.V.)	36			.13– .93	.44 ± .04		
III. A. Pregnant women at term with some vit. B_6 medication (M., V_1)	69	19–45	27	.06– .44	.20 ± .01	M., V_1 *vs* C., V_1	<.01
B. Cord blood (C., V_1)	61			.12–1.02	.47 ± .02	M., N.V. *vs* M., V_1	<.05
IV. A. Pregnant women at term with controlled vit. B_6 medication (M., V_2)	26	16–41	27	.04– .61	.28 ± .03	M., V_2 *vs* C., V_2 M., N.V. *vs* M., V_2	<.01 <.01
B. Cord blood (C., V_2)	22			.18–1.24	.55 ± .05	C., N.V. *vs* C., V_2	<.04

0.01 and 0.36, averaging 0.16 ± 0.01 mγ per million cells. The third experimental group was composed of 69 women at term who had occasionally taken Vit. B_6 containing preparations. Their pyridoxal phosphate level ranged between 0.06 and 0.44, averaging 0.20 ± 0.01 mγ/million cells. The 26 subjects in the fourth group had taken Vit. B_6 regularly for at least 14 days prior to delivery. Their blood level varied between 0.04 and 0.61 and averaged 0.28 ± 0.03 mγ B_6-al-PO_4/million leukocytes. The difference in the vitamin level between the control group and the pregnant group without vitamin supplementation is highly significant. Occasional intake of Vit. B_6 led to a moderate increase of leukocytic B_6 of doubtful significance. However, the level of the coenzyme form of Vit. B_6 is significantly increased in those women who ingested pyridoxine regularly. The vitamin content of their leukocytes approached that of the normal control group.

The amount of leukocytic pyridoxal phosphate was significantly greater in the cord blood as compared to the maternal blood in all groups examined. The cord blood of 26 infants borne by the women without vitamin supplementation contained between 0.13 and 0.93 (average 0.44 ± 0.04) mγ B_6-al-PO_4/million leukocytes, and that of 61 infants of women who had occasionally taken the vitamin between 0.12 and 1.12 (average 0.47 ± 0.02) mγ B_6-PO_4/million leukocytes. The highest level was found in the last group in 22 infants of mothers supplemented by vit. B_6. The levels of pyridoxal phosphate in these infants ranged between 0.18 and 1.24 mγ with an average of 0.55 ± 0.05 mγ/million leukocytes. The difference between the cord blood values of babies whose mothers were not Vit. B_6 supplemented and those who received the vitamin is statistically significant.

Comment. A decrease of circulating Vit. B_{12} similar to that of Vit. B_6 was recently described in the serum of pregnant women by Okuda *et al.*(6) and by Baker *et al.*(7). Okuda and his coworkers also found much higher Vit. B_{12} levels in the cord blood as compared to maternal blood. The concentration of glutamic-aspartic transaminase, a Vit. B_6 dependent enzyme, is much greater in the fetal than in the maternal blood(8). On the other hand, Friedman *et al.*(9) found no difference in amount of Vit. B_6 in blood, urine or skin of non-pregnant controls and women at term. This might, however, be explained by

difficulties with the bioassay used by these investigators and by the fact that all forms of the vitamin, metabolically active as well as inactive, were measured.

Present evidence indicates that Vit. B_6 is a very important nutrient that is essential for the human being(10). The central nervous system of the newborn infant appears to be particularly sensitive to a conditioned Vit. B_6 deficiency(11). The importance of Vit. B_6 for reproduction(12) and for normal fetal and infant development(13) has also been proven in the experimental animal. Obviously pregnancy exacts large demands on the maternal store of Vit. B_6. The results here reported suggest that the growing fetus may attract maternal Vit. B_6 across the placental barrier. It is possible to increase the amounts of circulating pyridoxal phosphate to almost normal control levels by oral administration of pyridoxine to pregnant women, and further to increase fetal levels, suggesting the probability of a placental transfer of the vitamin. It is interesting to note that a daily dose of 10 mg pyridoxine was found previously to rectify the abnormal tryptophan load tests in pregnant women. Supplementation of the normal diet by a daily dose of 10 mg pyridoxine hydrochloride has been suggested, and preliminary results have been reported indicating that such a regime may be of benefit to pregnant women, since there was a significant decrease of preeclampsia in a vitamin-supplemented as compared to a similar untreated control group(14).

Summary. Comparison of the pyridoxal phosphate content of circulating leukocytes in non-pregnant controls, women at term and umbilical cord blood shows significantly higher values in normal controls as compared to women at term. The cord blood shows the highest levels. Pyridoxine administration elevates the B_6-al-PO_4 levels in pregnant women to a very marked, and that of cord blood to a moderate, degree. It is concluded that the growing fetus can successfully compete with the mother for this important vitamin.

1. Wachstein, M., and Gudaitis, A., *J. Lab. and Clin. Med.,* 1952, v40, 550, and 1953, v42, 98.

2. Wachstein, M., and Lobel, S., PROC. SOC. EXP. BIOL. AND MED., 1954, v86, 624.

3. Wachstein, M., *Am. J. Clin. Nutr.,* 1956, v4, 369.

4. Boxer, G. E., Pruss, M. P., and Goodhart, R. S., *J. Nutrition,* in press.

5. Umbreit, W. W., *The Vitamins, Chemistry, Physiology, Pathology,* edited by Sebrell, Jr., W. H., and Harris, R. S., Academic Press, N. Y., 1954, v3, 239.

5a. Li, J. G., and Osgood, E. E., *Blood,* 1949, v4, 670.

6. Okuda, K., Helliger, A. E., and Chow, B. F., *Am. J. Clin. Nutr.,* 1956, v4, 440.

7. Baker, H., Erdberg, R., Pasher, I., and Sobotka, H., PROC. SOC. EXP. BIOL. AND MED., 1957, v94, 513.

8. Glendening, M. B., Cohen, A. M., and Page, E. W., *ibid.,* 1956, v90, 25.

9. Friedman, B. T., Becker, L., Thompson, C., and Vilter, R. W., *J. Lab. and Clin. Med.,* 1955, v46, 817.

10. Vilter, R. W., *J.A.M.A.,* 1955, v159, 1210.

11. May, Ch. D., *Pediatrics,* 1954, v14, 269.

12. Nelson, M. M., and Evans, H. M., *J. Nutr.,* 1951, v43, 281.

13. McGanity, W. J., Tucker, R. G., Turner, T. B., Utley, M. G., and Darby, W. J., *Fed. Proc.,* 1955, v14, 444.

14. Wachstein, M., and Graffeo, L. W., *Obstet. and Gynecol.,* 1956, v8, 177.

Received July 11, 1957. P.S.E.B.M., 1957, v96.

Respiratory Effects of Increased Air Flow Resistance in Dogs.* (23469)

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Following description of pneumotachographic technics by Silverman *et al.*(1) numerous investigators have studied the effects of resistance to air flow on patterns of breathing. Recently Zechman *et al.*(2) has shown that the primary effect of resistance to air flow is a reduction in air flow velocity and an increase in the duration of the impeded phase resulting in a reduction of pulmonary ventilation, a rise in alveolar CO_2 pressure and a fall in oxygen saturations. It was thought worthwhile to continue the study of impedances to air flow in anesthetized animals where greater impedances could be imposed and more extensive blood studies could be made.

Methods. Twenty-two mongrel dogs were used in these experiments. Weights ranged from 11 to 16 kg. It was not considered desirable to do all of the tests on each dog because of the great stress imposed on the individual animal. The number of dogs used in each series is indicated on each figure and table. Dogs were anesthetized by intraperitoneal injection of 55 mg of Dial in Urethane (Ciba)/kg body weight. A tracheal cannula containing a wire cloth flowmeter capsule for recording respiratory flow patterns and intratracheal pressures was inserted in the trachea. Various size orifices were inserted in the capsule. The capsule assembly added no significant volume to the respiratory dead space. Differential pressures across the flowmeter capsule were linear with flow within the range of values obtained. Thus tidal volume could be determined by planimetering the areas of the recordings and from these values pulmonary ventilation was calculated. Maximum inspiratory flow of air into the trachea could also be measured. A photograph of the assembly is shown in Fig. 1. Blood pressure was recorded by Statham transducers attached to polyethylene catheters 2 mm inside diameter. The free end of one of these catheters was inserted well up into the femoral artery. Blood samples were also drawn through this catheter. Blood samples were analyzed for oxygen content, oxygen capacity and carbon dioxide content by use of a Van Slyke manometric gas analyzer(3) and pH was measured with Cambridge Instrument Co. research type glass electrode. Blood samples were drawn anaerobically in 5 ml syringes and analyses made at once. Total hemoglobin was determined by converting hemoglobin in the samples to met-cyanhemoglobin and reading the concentration on an Evelyn photoelectric colorimeter. The factor of 1.35 was used to calculate total hemoglobin oxygen capacity. Femoral artery blood pressure, tracheal pressure and velocity of air flow into and out of the trachea were recorded simultaneously on a Miller Oscillograph. Recordings were made before and after orifices of various sizes were inserted in the tracheal cannula. Each dog breathed with an open tracheal cannula for 12 minutes and then an orifice was inserted. Breathing continued through it for 12 minutes and then recovery was permitted through an open cannula for

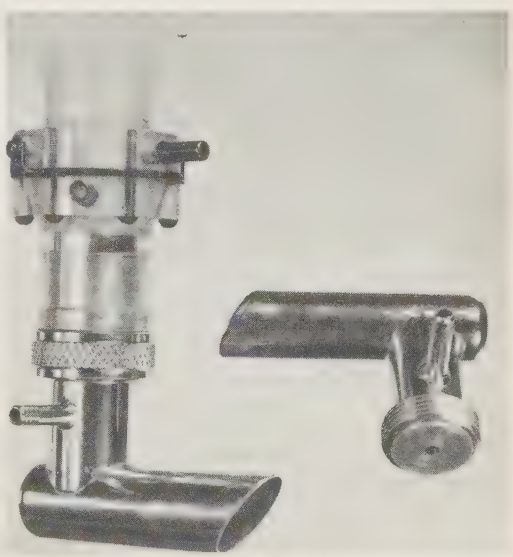

FIG. 1. Tracheal cannula and flow meter assembly showing position of plate orifices.

* Supported in part by Air Force Contract.

12 minutes before the next sized orifice was used. The results reported are from determinations made between the 11th and 12th minute. In preliminary tests it was found that orifices greater than 5 mm in diameter had little effect upon the factors under study. Pressure-flow characteristics were determined for each orifice using each respiratory gas used in experiments. Consideration was given to the fact that respiratory gases varied in density which in turn would affect the resistance of each orifice. However, this was found not significant enough to influence results. Four orifices were used each being 1 mm in thickness and having diameters of 1.5, 1.9, 2.3, 3.2 mm respectively.

Results. Mean values for various pressures and respiratory factors are plotted in Fig. 2 and 3. Points on the abscissas are shown as the reciprocals of the areas of the various orifices used, the open tracheal cannula being considered normal (N). Plotting points as reciprocal of the areas is somewhat arbitrary for flow through orifices of these dimensions cannot accurately be expressed so simply. These graphs are used solely to illustrate the relative manner in which different factors vary relatively with varying obstruction to breathing.

Table I shows how arterial oxygen saturation and CO_2 pressures change with obstruction to breathing. It will be noted that only when very small orifices are used to obstruct breathing does arterial oxygen saturation fall to a low level. Fig. 4 shows the effect of the

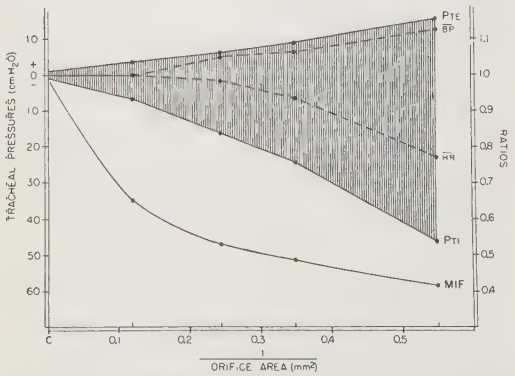

FIG. 2. Influence of air flow impedance on tracheal air flow and pressure, arterial blood pressure and heart rate.

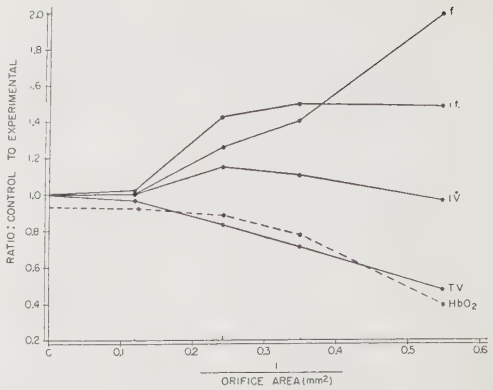

FIG. 3. Influence of air flow impedance on respiratory frequency, inspiratory time, minute volume, tidal volume and arterial oxygen saturation.

various orifices on the oxygen and carbon dioxide pressures. The tracheal pressure relations obtained are shown in Fig. 2. PTE represents average maximum positive tracheal pressures recorded during expiration in cm H_2O and PTI represents average maximum negative pressures recorded during inspiration. BP represents average maximum systolic blood pressures recorded and HR represents average heart rate recorded. MIF represents average maximum instantaneous flow of air into the trachea on inspiration. The latter 3 factors are expressed in ratios, relating the values obtained while dogs were breathing through resistances to the control values while breathing through an open trachea. Thus each dog was his own control and average values can be shown in a single figure which expresses the results in a summary manner.

It should be kept in mind that in these experiments resistance to breathing was the same for inspiration as for expiration and

TABLE I. Summary of Oxygen Saturation and pCO_2 Values for Arterial Blood of 10 Dogs after Breathing through Various Size Orifices for 12 Min.

Orifice area	% Hb O_2	pCO_2, mm Hg
Open trachea	93 ± 2.6*	44 ± 3.0*
25 mm²	93 ± 4.0	44 ± 3.2
8.3	92 ± 3.6	45 ± 3.0
4.1	89 ± 7.6	51 ± 6.2
2.8	78 ± 14.0	59 ± 9.7
1.9	39 ± 23.4	69 ± 10.9

* ± S.D.

TABLE II. Tidal Air, Breathing Rates and Maximum Tracheal Pressures (Avg for 6 Dogs Breathing through 1.9 mm Diameter Orifice).

	Maximum tracheal pressures			
	Inspir., cm H_2O	Expir., cm H_2O	Tidal air, ml	Breathing rate/min.
Control (open trachea)	.1	.7	164	16
Breathing air	19.2	6.3	122	21
" 100% O_2	17.0	5.1	147	11
" 5.6% CO_2 (in oxygen)	20.9	7.8	129	21

also that pressure-flow relations are not linear. Of course the tidal volume must be moved twice in each breathing cycle, once with negative pressure and once with positive pressure, and the tidal volume must be moved within a limited time if alveolar ventilation is to be maintained at an adequate level. It would appear that this is accomplished almost entirely by an increased frequency of breathing (see f, Fig. 3). A point was finally reached, however, with the smallest orifice when pulmonary ventilation (see IV, Fig. 3) was inadequate to maintain proper alveolar pO_2 and pCO_2 levels and respiratory failure ensued. It is interesting to note that this orifice with an area of 1.8 mm^2 was less than one-hundredth the area of the trachea. The relative length of time of inspiration during the breathing cycle increased when small orifices were inserted but this is of little advantage to the animals since the tidal volume must be moved in and out of the lungs in each cycle against similar resistances. Also shown in Fig. 3 are other respiratory factors which are influenced by breathing through the orifices of various sizes: i.t. represents relative length of time of the breathing cycle taken by inspiration; IV, pulmonary ventilation or minute volume; TV, tidal volume for each inspiration; HbO_2, percentage oxygen saturation of arterial hemoglobin. These are mean values obtained on 7 dogs, each dog was his own control (C) and 1.0 is the control value for the resting condition with each dog breathing through a wide open trachea. It can be seen that oxygen saturation values decreased in proportion to decreases in tidal volume.

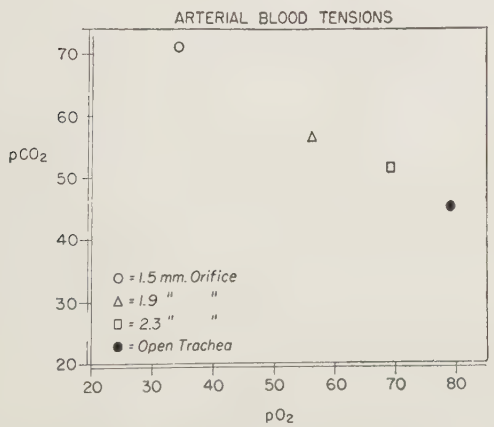

FIG. 4. Effect of different plate orifices on arterial oxygen and carbon dioxide tensions.

To gain a better understanding of the control of respiration when increased air flow resistance is encountered, a separate experimental series was conducted. Each dog was used as his own control, *i.e.*, recordings were made on each dog while it was breathing through an open trachea, then a 1.9 mm diameter orifice was inserted in the tracheal cannula and subsequent measurements were made while each dog breathed gas mixtures as indicated. Only one orifice was used in order to reduce total stress of breathing to a minimum.

The results are summarized in Tables II and III. Breathing 5.6% CO_2 in oxygen produced the highest tracheal pressures and breathing 100% oxygen the lowest, but the differences are small. The more striking ef-

TABLE III. Average Arterial Gas Tensions in 6 Dogs Breathing through 1.9 mm Diameter Orifice.

	Hb O_2 (%)	p CO_2 (mm Hg)
Control	90	43
Breathing air	77	51
" 100% O_2	99	58
" 5.6% CO_2 (in oxygen)	97	65

fects obtained were on breathing rate. There was a pronounced decrease in breathing rate while dogs were breathing 100% oxygen.

It was stated(2) that increased air flow resistance in humans resulted in a decreased respiratory frequency. In the human studies, unlike these on dogs, the level of resistance was not sufficient to produce hypoxia. The decreased frequency of dogs breathing 100% oxygen seems to indicate that increased air flow resistance *per se* decreases breathing rate, but if severe enough to cause hypoxia, an increase in rate is initiated. The pause between expiration and inspiration in the normal dog is retained while breathing oxygen through resistances, but is lost while breathing air in an hypoxic state. Hypoxia eliminates or greatly reduces the respiratory pause.

Conclusions. 1. When tracheal air flow is so impeded as to produce pronounced changes in inspiratory and expiratory pressures, breathing rates increase, minute volumes may remain relatively uniform and tidal volumes decrease inversely with the pressure differences. 2. Two factors are involved in the accommodation to this respiratory stress: (a) Increasing carbon dioxide tensions act as primary stimuli to more forced inspiration and expiration; (b) Decreasing oxygen tensions act as stimuli to more rapid breathing by reducing the time of the expiratory phase of the breathing cycle.

1. Silverman, L., Lee, R. C., and Drinker, C., *J. Clin. Invest.*, 1944, v23, 907.

2. Zechman, F., Hall, F. G., and Hull, W. E., *J. App. Physiol.*, 1957, v10, 356.

3. Van Slyke, D. D., and Neill, J. M., *J. Biol. Chem.*, 1924, v61, 523.

Received July 15, 1957. P.S.E.B.M., 1957, v96.

Presence of a Carcinogenic Substance in Hens' Eggs. (23470)

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It was shown previously that hens' eggs contain a substance that stimulates growth and sex development. Chicks maintained on a diet made up of eggs and wheat bran grew faster and became sexually mature earlier than the control chicks of the same breed and age maintained on a Purina broiler mash diet. The females became productive relatively early and the males developed very large combs, wattles and spurs. Females maintained on the above diet for over 2 years developed cancer of the ovary(1). Recently Menge, Lillie and Denton(2) reported the presence in egg yolk of a growth stimulating factor. The authors state that the latter is destroyed by heat, since ashes of yolk are without any effect upon growth. In the experiments carried out by the author(1) the eggs used in the diet were autoclaved for at least 30 minutes at 15 lb pressure. It is possible, however, that the growth-promoting factor in this case is the same as that reported by Menge and his co-workers; the moist heat of the autoclave may not be as destructive as ashing. There is also the possibility that these are 2 different substances, since the egg diet stimulated sex development in addition to growth. More work will have to be done in order to identify the growth-stimulating substance or substances in eggs. Since chicks were the only animals used in the study of the growth-stimulating action of eggs, the question arose whether animals other than birds would be affected in their growth by a diet containing eggs and to what extent such a diet might influence the development of malignant tumors. These were the bases for a new series of experiments in which mice were used. The present is a report of the first series of experiments carried out with "A" strain mice.

Methods. Sixteen mice (8 males and 8 females caged separately) were placed on a Rockland rat diet supplemented with eggs.

TABLE I. Time of Appearance, Location and Identity of Malignancies in the Experimental Mice.

Animal No.	Sex	Age in mo	Months on diet	Primary tumors	Location	Identity of tumor	Distant metastases
1	♂	9	6	1	periton.	sarcoma	abd. wall
6	"	15	12	1	lungs	adenocarcinoma	none
7	"	19	16	1	"	"	"
8	"	20	17	1	"	"	"
9	♀	20	17	1	"	"	"
10	"	21	18	1	mammary gl.	"	"
11	"	22	19	2	lungs	"	"
					ovary	"	peritoneum
12	"	22	19	3	lungs	"	none
					ovary	"	"
					mammary gl.	"	peritoneum
13	♂	22	19	2	leg	osteosarcoma	ribs
					lungs	adenocarcinoma	liver
14	"	24	21	2	lungs	"	none
					lymphoid	leukemia	
15	♀	24	21	4	lungs	adenocarcinoma	none
					ad-cort.	"	"
					ovary	sarcoma	peritoneum
					lymphoid	leukemia	
16	"	25	22	3	lungs	adenocarcinoma	none
					ovary	cystoadenoma	peritoneum
					lymphoid	leukemia	

The eggs (one or 2 fresh eggs daily) were boiled for 20 minutes. They were then split into halves and one half placed in the cage containing the males and the other in that of the females. The mice usually eat the yolk, leaving the white intact. Sixteen other mice (8 males and 8 females) from the same litters as the above were used as controls. They were maintained on a Rockland rat diet only; no eggs were given to them. In this case also the males and females were kept in separate cages. The duration of the experiment was 22 months. Most of the experimental mice died 12 to 18 months after they were put on the egg diet. The experiment was not concluded, however, until the last experimental mouse died. Of the control mice, 6 died at the age of 15 to 20 months, the remaining 10 were killed at the age of 25 months, when the last experimental mouse died. All the animals, experimental and controls, were autopsied and any diseased organs fixed, sectioned and studied microscopically.

Results were as follows: of the 16 controls, only 2 mice developed cancer. Both were females. One died at the age of 20 months, and the other was killed at the age of 25 months. In both cases an adenocarcinoma of the lungs was found. The tumor invaded both lungs, but it did not metastasize outside of the respiratory system. Of the 16 experimental mice, 12 had malignancies at the time of death. In 6 of these mice, which died 6 to 18 months after they were put on the egg diet, only one type of malignant tumor was found in each (mice nos. 1, 6, 7, 8, 9 and 10, as shown in Table I). In the other 6 mice, which lived a little longer, 2, 3, and even 4 different malignancies were found in each of them. Moreover, in the latter group distant metastases were frequent, whereas in the first group the malignancy remained localized in the organ in which it developed.

Adenocarcinoma of the lungs appears to be the most frequent malignancy in the experimental animals, but "A" strain mice are known to spontaneously produce this type of tumor. There is, however, a great difference in the rate of appearance of this malignancy between the mice on the diet supplemented with eggs and the controls. Among the latter group only 2 mice were found to have lung tumors, while of the 16 experimental animals, which were from the same litters as the above, 10 developed malignancies in the respiratory system. The lung adenocarcinomas of the

different animals were histologically similar, although they did not seem to be of the same origin. Some appeared to have developed from the bronchial epithelium, while others, without any doubt, originated from the lung parenchyma. In all of the animals with the above malignancy, the tumors were multiple; whether they were all primary or some were secondary is not known. In one case (animal 13) the lung tumor metastasized to the liver. In this case the mitotic activity of the tumor was considerably higher than in the other lung tumors. Moreover, it is interesting that this animal, in addition to its lung adenocarcinoma, was affected by a highly active osteosarcoma. A similar phenomenon was observed in other animals with multiple primary malignancies in which distant metastases of one or even of 2 of them were found. The presence of one primary malignant tumor, consequently, does not interfere with the growth and propagation of another one.

The females were all virgins, yet 2 of them developed mammary gland adenocarcinomas. These tumors were rapidly growing and one of them (animal 12) metastasized to one of the lungs and into the peritoneal cavity, close to the kidney. This animal, in addition to its mammary tumor, had a highly active primary lung adenocarcinoma and a cystadenoma of the ovary. Cysts of ovarian origin were found, in this case, in the peritoneal cavity at a distance from the ovary.

In 3 experimental animals sarcomas of different types were found. In one of them (animal 1) the malignancy, a spindle-cell sarcoma, appeared during the 6th month of the experiment. It was considered to be of peritoneal origin because of the large mass it formed in the abdominal cavity, surrounding all the viscera and invading the pancreas and part of the liver. The muscles of the abdominal wall and the subcutaneous tissue were also in part invaded by the tumor. A large mass of necrosed fat was found in this case in the peritoneal cavity and subcutaneously. It was probably caused by enzymes liberated from the pancreas, which was invaded and partially destroyed by the tumor.

In animal 13 the osteosarcoma, with its characteristic giant cells, was a rapidly growing tumor. It consisted of a relatively large mass on the left leg and many smaller nodules upon the chest wall on each side of the sternum. The former was considered as the primary and the latter as secondary tumors, but it is possible that all of them are primary growths. Histologically, no difference in structure nor in the rate of growth could be found between them. All of these tumors were continuous with the bones of the corresponding regions. As mentioned above, this animal, in addition to its sarcoma, had a lung adenocarcinoma which metastasized to the liver.

Finally, in animal 15 the picture was quite complicated. It had an adenocarcinoma of the lungs, an adenocarcinoma of the adrenal gland (the cortex), a lymphatic leukemia (characterized by very much enlarged spleen and lymph nodes, and by the infiltration of the liver, kidneys and circulatory system with lymphocytes) and a round cell sarcoma of the ovary. The latter consisted of a large mass of round cells surrounding a few degenerated follicles. Another tumor, of the size of a small cherry, was found in the lower part of the abdominal cavity of the same animal. It was identified as a mixed-cell sarcoma of ovarian origin, but it may well have developed independently from the peritoneum. The cortical adrenal adenocarcinoma, in this mouse, consisted of a large mass of anaplastic cells with no typical organization. The medulla was completely destroyed and the tumor had invaded the capsule of the kidney, but no distant metastases were found.

The ovaries were abnormal in all of the experimental females. In some of them the follicles were either in the process of degeneration or completely absent, and in 4 mice ovarian tumors were present. In animal 11 the ovary consisted of a mass of undifferentiated cells, giving the appearance of an adenocarcinoma. In this animal cysts of ovarian origin were found in the peritoneal cavity at a distance from the reproductive organ. An adenocarcinoma of the ovary was also found in animal 12, but in this case no distant metastases were present. In animal 15, as mentioned previously, a round-cell sarcoma replaced the ovary. Finally, in animal 16 the ovary con-

sisted of a small mass of undifferentiated cells and a large number of cysts. One large cyst was found in the peritoneal cavity, without any connection with the ovary. Some of these cysts were filled with clear fluid, while others contained a yellow, viscous substance. In the females with ovarian tumors the epithelial lining of the oviduct had undergone metaplasia. No damage to the testis could be found in any of the males. A similar observation was made previously in connection with chicks maintained on an egg diet(1).

The spleen and the lymph nodes were considerably enlarged in many of the experimental animals. In 3 of them, which were identified as leukemic, in addition to the large nodular spleen and the enlarged lymph nodes, the liver, kidneys and circulatory system were invaded by lymphocytes.

The liver underwent important changes in the experimental animals. Its cells very often lost their cohesion. They became vacuolated, and a great number of them were binucleated. The nuclei in the liver cells were irregular in size, and the nucleoli abnormally large. The liver picture was particularly abnormal in animals 10, 12, 14, 15 and 16, in which numerous cells were in the process of necrosis, while other groups of cells were in mitosis. Among the latter a great number of cells were binucleated or multinucleated. In some places the liver lobules consisted of a few isolated cells, while in other places no lobular structure could be recognized. It is possible that there is some relationship between the liver damage and the development of the malignant tumors, but for the time being it is too early to draw any conclusion on the subject.

Another organ which seems to be affected by the egg diet is the pancreas, but the general organization of the gland does not change.

Summary. Sixteen "A" strain mice were maintained, from the age of 3 months, on a Rockland rat diet supplemented with hard boiled eggs, one or 2 daily. Sixteen other mice, from the same litters as the above, were kept as controls. They were maintained on the same Rockland rat diet, but without eggs. Of the latter group only 2 animals developed tumors, which were in the lungs, one at 20 and the other at 25 months of age. Of the 16 experimental mice, 12 developed malignant tumors. In 6 of them the tumors in each animal were limited to a single organ, while of the other 6 mice, which died at the age of 22 to 25 months, some had 2, others 3 and one had 4 primary malignancies. Adenocarcinoma of the lung was the most frequent malignancy. It was found in 10 of the experimental animals. Next in frequency were ovarian tumors, leukemias, mammary gland adenocarcinomas, an osteosarcoma and an adrenal gland adenocarcinoma. In many of these animals distant metastases were found. In the experimental females the ovaries underwent radical changes. Four of them became cancerous. In none of the males, however, could any damage be found in the testes. It is too early to draw definitive conclusions, but there seems to be no doubt that eggs contain a substance or substances that stimulates both normal and abnormal growth.

1. Szepsenwol, J., *VI Congres Fed. International d'Anat.*, Paris, 1955, 213.

2. Menge, H., Lillie, R. J., and Denton, C. A., *Fed. Proc.*, 1957, v16, 1682.

Received July 15, 1957. P.S.E.B.M., 1957, v96.

Propagation of Conjunctival and HeLa Cells in Various Carbohydrate Media.* (23471)

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Establishment of epithelial-like cells in continuous culture from human tissues has made available a more effective system for the study of metabolism of human cells *in vitro* than the previously used tissue explant method. Under controlled experimental conditions, using media of relatively well defined composition, these established cells may be maintained in a state of active multiplication and the rate of multiplication can be measured quite accurately. This report deals with propagation of these human cells in the presence of certain individual carbohydrates.

Materials and method. Human cells. Conjunctival(1) and HeLa cells(2) were used as representative cell lines, the former derived from normal and the latter from malignant human tissues. Stock cultures were propagated in 20% inactivated human serum diluted in Eagle's basal medium(3). Cultures were checked at regular intervals, and also during each critical experiment, to assure freedom from contamination by pleuro-pneumonia-like organism(4) and bacteria. *Media.* Basal carbohydrate-free medium consisted of 10% dialyzed serum in a modified Eagle's basal medium; the modifications consisted of elimination of glucose, addition of meso-inositol to final concentration of 10 to 20 micromolar, and the substitution of Earle's balanced salt solution by Hanks'. Carbohydrates being tested were added to this medium to final concentrations of 5 mM. *Sera* were collected and stored at −60°C as described (5). Dialyzed serum was prepared by dialysis of about 100 ml serum containing about 100,000 units of penicillin and 10 mg of streptomycin against 10 changes of 0.85% NaCl of 1 liter each at about 2°C for 48 hours. Sera so dialyzed were negative for glucose by the glucose oxidase test. *Glucose oxidase test.* The enzyme paper stick method(6) was used, and the final reading was made exactly 1 minute after application of test solution. The minimum concentration of glucose which gives a positive reaction is about 0.05 mM (0.5 to 1.0 mg per 100 ml). *Carbohydrates.*† Included in the present study were soluble starch, glycogen, maltose, sucrose, lactose, d-fructose, d(+)galactose, l(+)rhamnose, l(−)fucose, l(−)sorbose, d-ribose, d(+)xylose, l(−)xylose, d(−)lyxose, l(+)arabinose, d(−)arabinose, d-2-deoxyribose, i-erythritol, dihydroxyacetone, sodium pyruvate and sodium lactate. When tested for presence of glucose as impurity by the glucose oxidase test, the following results expressed in terms of highest concentration giving a negative test were obtained: Starch‡ 0.02 M, glycogen‡ 0.02 M, maltose 0.02 M, lactose 0.3 M, d(+) galactose 0.01 M (this preparation was specified as "glucose free"), d-mannose 0.03 M, d(+) xylose 0.5 M and all others negative at saturation (more than 1 M). Since, as will be shown, glucose is effective in supporting cell propagation at 0.05 to 0.1 mM, it is essential that the glucose present in starch, glycogen, maltose, d(+) galactose and d-mannose be removed. The amount of glucose in lactose and d(+)xylose was too small to be of consequence in the present studies. Tenth molar starch and glycogen solutions were dialyzed against running water for about 24 hours at 0-2°C; these dialyzed polysaccharide solutions, after passage through bacterial filters, no longer gave positive glucose oxidase tests. Maltose, d(+)galactose and d-mannose were treated with the enzyme, glucose oxidase

* This investigation was supported in part by research grants from Natl. Inst. of Microbiology and Cancer, P.H.S., and the Amer. Cancer Soc.

† Starch and sodium lactate were purchased from Mallinckrodt Co.; lactose, maltose and sucrose, from Merck; d(+)galactose and d(+)xylose, from Pfanstiehl; d-fructose and d-mannose, from Eastman Kodak; and, all the others from Nutritional Biochem. Corp.

‡ Concentrations of all polysaccharides were based on glucose.

(Glucostat, Worthington Biochemical Corp.); about 150 mg of enzyme were used for each 10 millimoles of carbohydrate. After aeration for 3 hours, the enzyme-carbohydrate mixture was dialyzed against a small volume of sterile water for 16-20 hours at 2°C. The amount of carbohydrate present in the dialysing fluid was determined by dry weight analysis; such determinations were performed after filtration through an ultrafine glass filter. Maltose so treated gave a negative glucose oxidase test at 0.8 M concentration, galactose at 0.15 M, and d-mannose at 0.05 M. This failure to reduce substantially the reaction of d-mannose and d(+) galactose with glucose oxidase may possibly be explained by the oxidation of these 2 carbohydrates in the presence of this enzyme(7).

Method of evaluation. About 20,000 cells suspended in 0.2 ml basal medium were inoculated into a series of 6 to 12 new culture tubes, 18 x 150 mm, containing 0.8 ml basal medium plus the carbohydrate to be tested. After slanting at 36°C for about 20 hours, the tubes were placed on a roller drum also at 36°C. Media were renewed on the 3rd and then on every other day. The number of cells/tube was counted, generally on 4th and 8th day; 2-4 tubes were used at each counting. To facilitate description, this will be referred to subsequently as the *direct inoculation method.* To eliminate possible unknown factors introduced through tryptic digestion and mechanical agitation (both are essential steps in the preparation of homogeneous cell inoculum), all carbohydrates incapable of sustaining cell growth by the direct inoculation method were further tested by a *medium replacement method.* One-half ml of basal medium containing 0.2 mM glucose and about 30,000 cells was inoculated into each of a large series of culture tubes. On the 3rd day, when sheets of polyhedral cells were formed, the average number of cells/tube was calculated from cell counts in any 4 tubes picked at random. After 3 successive washings with basal carbohydrate-free medium, the nutrient medium in remaining tubes was replaced by the basal medium plus a carbohydrate to be tested; this was then considered as the initial day of the test period. Six tubes were generally used for each carbohydrate. Media were renewed every second day. The number of cells/tube was determined when definite degeneration appeared in the carbohydrate-free controls, and, again when degeneration was completed. Technical details of a similar quantitative study have already been described(5). In this particular study, reagents such as trypsin and antibiotics were dissolved in 0.85% NaCl rather than the balanced salt solution which contained glucose; the cell inoculum was washed twice in 10 ml carbohydrate-free medium; and, 2 controls, the basal carbohydrate-free and the basal plus 5 mM glucose, were included in each experiment. No attempt was made to evaluate minor differences in growth rate. Adaptive changes will be considered in another communication.

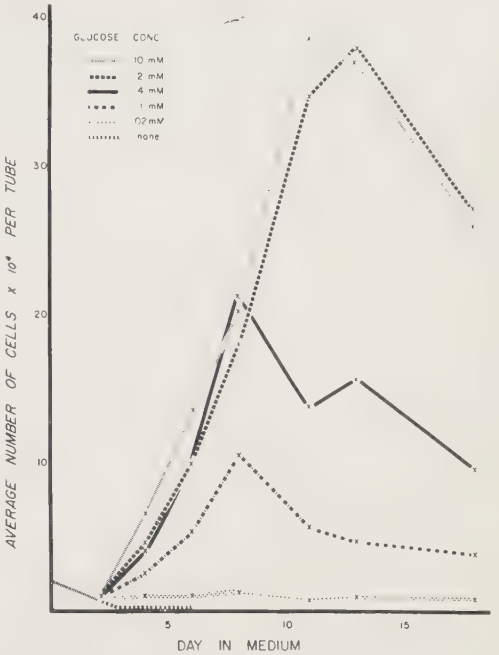

FIG. 1. Propagation of conjunctival cells in various concentrations of glucose.

Results. Cell growth in various concentrations of glucose. To provide basic data for subsequent comparison, multiplication patterns of the conjunctival cell in medium containing glucose at final concentrations of 10, 2, 0.4, 0.1, 0.02, 0.004, and 0.0 mM were used. Results are presented in Fig. 1. Similar growth curves were obtained in media containing glucose at concentrations of 10 and 2

TABLE I. Propagation of Conjunctival Cells in the Presence of Individual Carbohydrates.*

Carbohydrate	Cells × 10^3/tube on the following days: 0	4th	8th
None	29	3	0
Glucose	29	104	158
Starch	29	95	143
Sucrose	29	19	43
d-ribose	29	9	0

* See text for pertinent data.

mM; both showed a lag phase of about 2 days, followed by logarithmic growth period of about 10 days, which terminated in partial cell degeneration after a maximum cell population of about 300,000/tube was reached. At 0.4 mM, partial cell degeneration appeared several days earlier when the maximum population of about 200,000 was attained. At 0.1 mM, the slope of the logarithmic growth phase was less steep and when the cell population was about 100,000/tube, partial degeneration became apparent. At 0.02 and 0.004 mM glucose, evidence of cell multiplication and degeneration were present after about the 3rd day, no logarithmic growth phase was observed, and cell populations were maintained at approximately 5,000 to 10,000/tube. In the basal carbohydrate-free medium, degeneration was completed by the 4th to 7th day. Minor variations were observed depending on the physiological state of cell inoculum, sera used and cell lines tested. These variations were in length of the lag phase, the maximum population attainable before onset of partial degeneration, and the number of days in the carbohydrate-free medium for completion of degeneration. It is therefore essential to compare only results obtained within a single experiment.

Both conjunctival and HeLa cells have been maintained and subcultivated in the basal medium plus glucose at a concentration of 0.1 mM for 2-3 months. At this low concentration of glucose, the net increase in number of cells/week is significantly lower than that in medium containing glucose at concentration of 10 mM. No evidence of partial degeneration could be observed when the cell population per 1 ml medium did not exceed 50,000. It seemed that the actual rate of multiplication of both cells may have been reduced appreciably at this low concentration of glucose.

Cell propagation in carbohydrate other than glucose. Results for the conjunctival cells by the direct inoculation method are presented in Table I. Starch, glycogen, maltose, d(+)galactose, d-fructose and d-mannose supported cell multiplication satisfactorily; the net increase in the number of cells in 8 days in the presence of these carbohydrates was similar to that with glucose at a concentration of 5 mM. Results obtained with sucrose were dependent on the dialyzed serum used. When tested with 2 dialyzed horse sera, results such as those presented in Table I were obtained. However, when 3 individual dialyzed human sera were used, sucrose failed completely to sustain cell growth. Lactose, l(+)rhamnose, l(−)fucose, l-sorbose, l(−)xylose, d(+)xylose, d(−)lyxose, d(−)arabinose, l(+)arabinose, d-2-deoxyribose; i-erythritol, dihydroxyacetone, sodium pyruvate and sodium lactate were unable to support cell propagation; the results were similar to those obtained with d-ribose, which are shown in Table I. Essentially similar results were obtained with the HeLa cells. Except in the case of sucrose, no appreciable difference in the results was obtained whether a dialyzed horse or a dialyzed human serum was used. Those carbohydrates incapable of sustaining cell growth were further tested by the medium replacement method. Results are shown in Table II. Sodium pyruvate, sodium lactate, d-ribose and d(+)xylose delayed the degeneration of both kinds of cells. L(+)rhamnose, l(−)fucose and l(−)sorbose appeared to retard the degeneration of the HeLa cells more effectively than the conjunctival cells. Lactose, l(−)xylose, d(−)lyxose, d(−)arabinose, l(+) arabinose, d-2-deoxyribose, i-erythritol and dihydroxyacetone failed completely to produce any beneficial effect.

Carbohydrases in dialyzed serum. The satisfactory cell propagation in such carbohydrates as starch, glycogen, maltose and sucrose may be explained by the existence of amylase, maltase and, perhaps, even sucrase in the serum. These enzymes would hydrolyze the poly- and di-saccharides into glucose

TABLE II. Degeneration of Conjunctival and HeLa Cells in Certain Selected Carbohydrates.

Carbohydrate	Cell × 10^3/tube on the following days: 0 Conj.	0 HeLa	4th Conj.	4th HeLa	8th Conj.	8th HeLa	20th Conj.	20th HeLa
None	33	26	2	22	0	0		
Glucose	33	26	132	112	217	397		
Pyruvate	33	26	9	14	7	11	5	7
l(+)rhamnose	33	26	2	20	3	7	0	6
d-2-deoxyribose	33	26	3	5	0	0		

and fructose, both of which are readily utilized by the cells. In several actual determinations, we were able to demonstrate positive glucose oxidase reaction with dialyzed serum containing starch, glycogen, and maltose after incubation of these serum-carbohydrate mixtures at 37°C for 16-20 hours; 2 individual dialyzed horse sera and 3 dialyzed human sera tested showed the ability of hydrolyzing these 3 carbohydrates. With sucrose, a positive glucose reaction was obtained when incubated with the dialyzed horse but not with the dialyzed human sera. No glucose was detected after incubation of lactose with any of the 5 dialyzed sera tested. This finding corresponded closely with the results of cell propagation studies using these dialyzed sera and carbohydrates. Satisfactory propagation occurred in starch, glycogen and maltose with any of the sera, sucrose would support cell growth only when dialyzed horse sera were used and lactose failed completely to support growth with all of the sera.

Glucose sparing effect of alanine and pyruvate. In view of the finding that sodium pyruvate was able to delay cell degeneration, the addition of this intermediate to medium containing a low concentration of glucose may spare some of the glucose from being oxidized as a source of energy, thereby increasing the amount of glucose available for synthesis of essential cell components. Results of experiments presented in Table III appeared to support this hypothesis. The addition of pyruvate and dl-alanine to media containing 0.1 mM glucose prevented the partial cell degeneration normally occurring between the 10th and 18th days. Cell multiplication occurred in media containing 0.01 mM glucose when pyruvate and alanine were added, while at 0.001 mM glucose, the addition of pyruvate and alanine delayed the rate of cell degeneration.

In an experiment with the conjunctival cells, when glucose was substituted by d-mannose or d(+)galactose, both at concentrations of 0.01 mM, satisfactory cell multiplication was observed in the presence of alanine and pyruvate; d-fructose was an effective substitute only at higher concentrations. This glucose sparing effect was shown to be due largely to pyruvate; alanine without pyruvate was completely ineffective; and, pyruvate without alanine was not as effective as pyruvate with alanine.

Discussion. Harris and Kutsky(8) reported that glycogen, maltose, d-mannose, d-fructose supported vigorous growth of chick heart fibroblast, that d-galactose was inhibitory and that sucrose, d-ribose and d-xylose were inert. Our results with human cells showed that d(+)galactose supported cell growth as well as glucose and that d-ribose and d-xylose retarded cell degeneration. Differences in the cell system used, the method of quantitation and the assay medium may account for some of these disagreements.

The presence of carbohydrases in serum

TABLE III. Glucose Sparing Effect of Pyruvate and dl-alanine.*

Medium† Glucose	Pyruvate	Alanine	Cells × 10^3/tube on following days: 0	6th	10th	18th
.1	0	0	14	67	158	38
.1	5	5	14	64	197	246
.01	0	0	14	3	4	0
.01	5	5	14	9	47	58
.001	0	0	14	0	0	
.001	5	5	14	3	1	0

* Results obtained with conjunctival cell. Tests with HeLa cells gave similar results.

† Basal carbohydrate-free medium with addition of glucose, pyruvate and dl-alanine concentrations indicated (expressed in mM).

may be an explanation for the satisfactory cell propagation in starch, glycogen, maltose and sucrose, as suggested by Harris(8). The ability of the 2 dialyzed equine sera and the inability of the 3 human sera to hydrolyze sucrose sufficiently to support cell growth tends to support this view.

The difference in the sucrase activities of the various individual sera is of interest in studies of the nutritional requirement of human cells. It illustrates how the results of certain studies may be influenced by the source of serum incorporated into the assay medium. Other differences of sera have already been described(9).

Summary. 1) Under the described experimental conditions, both conjunctival and HeLa cells propagated satisfactorily in the following individual carbohydrates: starch, glycogen, maltose, d-glucose, d-fructose, d-mannose and d(+)galactose. Cell propagation in sucrose depended largely on source of dialyzed serum. Sodium pyruvate, sodium lactate, d-ribose and d(+)xylose were unable to support cell multiplication, though cell degeneration in the presence of these carbohydrates and intermediates was definitely retarded. L(+)rhamnose, l(−)fucose and l-sorbose appeared to delay the degeneration of the HeLa cells more effectively than that of the conjunctival cells. Lactose, l(−)xylose, d(−)arabinose, l(+)arabinose, d-2-deoxyribose, i-erythritol and dihydroxyacetone neither supported growth nor retarded degeneration. 2) Glucose at low concentration of 0.1 mM is capable of supporting cell growth for at least several months. Net increase in number of cells at a concentration of 0.1 mM is significantly lower than that in 10 mM glucose. When pyruvate and alanine were added to the medium, the concentration of glucose could be further reduced to 0.01 mM without resulting in cell degeneration. D-mannose and d(+)galactose both at 0.01 mM concentrations, and d-fructose at somewhat higher concentration, are effective substitutes for glucose.

The authors are grateful to Dr. J. C. Snyder for his interest and suggestions in this work.

1. Chang, R. S., PROC. SOC. EXP. BIOL. AND MED., 1954, v87, 440.

2. Gey, G. O., Coffman, W. D., and Kubicek, M. T., *Cancer Research,* 1952, v12, 2164.

3. Eagle, H., *J. Exp. Med.,* 1955, v102, 37.

4. Robinson, L. B., and Wichelhausen, R. H., *Science,* 1956, v124, 1147.

5. Chang, R. S., and Geyer, R. P., *J. Immunol.,*

6. Adams, E. C., Burkhart, C. E., and Free, A. H., *Science,* 1957, v125, 1082.

7. Keilin, D., and Hartree, E. F., *Biochem. J.,* 1948, v42, 221.

8. Harris, M., and Kutsky, P. B., *J. Cell. Comp. Physiol.,* 1953, v42, 449.

9. Chang, R. S., *Conf. on Virus, Nucleic Acids and Cellular Biol.,* N. Y. Acad. Sciences.

Received July 18, 1957. P.S.E.B.M., 1957, v96.

Effect of Beta-Aminopropionitrile on Incorporation of Glycine by Croton Oil Pouches in Rats.* (23472)

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Although several investigators have studied the influence of beta-aminopropionitrile (BAPN) on mesodermal tissues, the mechanism of the BAPN effect is still not understood. Dasler(1) pointed out that it is tissues which are high in collagen that appear to be primarily affected by the lathyrus factor, and he has suggested that lathyrism involves a disturbance in the metabolism of connective tissue or of a connective tissue component. Ponseti and Shepard(2) also concluded that mesenchymal tissues are influenced by the lathyrus

* This research was supported in part by funds from the U. S. Public Health Service.

factor, the active principle of which is BAPN (3).

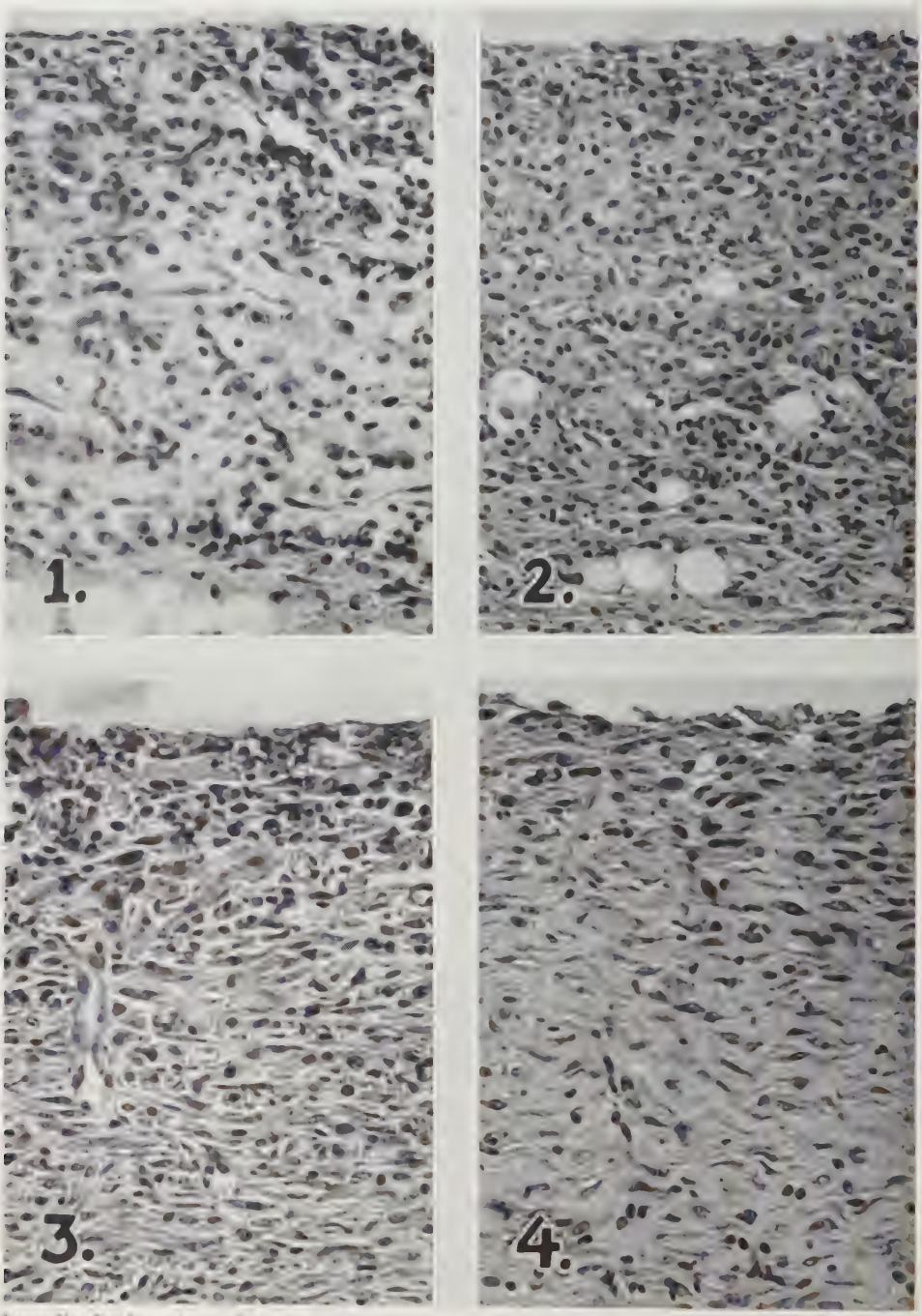
A previous study carried out in this laboratory has shown that the use of Selye's croton oil pouch technic provides an excellent tool for demonstrating the influence of BAPN on newly formed connective tissue(4). This technic has been used in the present investigation to determine the effect of BAPN on incorporation of radioactive glycine into croton oil pouches and dermis of rats.

Methods. Croton oil pouches were produced in female Sprague-Dawley rats according to a modification of Selye's procedure(5). Rats weighing 80 to 90 g were injected subcutaneously in the dorsal region with 20 ml of air followed by 1 ml of 1% croton oil. *In vivo* studies using radioactive glycine were carried out on 12 rats with croton oil pouches. Six test rats were fed 40 mg of mono beta-aminopropionitrile fumarate† (BAPN) in 20 ml of drinking water daily for 9 days, while control rats received no BAPN. A commercially prepared rat pellet diet‡ was fed during the course of these studies. All animals were weighed at the beginning of the experiment and again before sacrificing them on the ninth day. On the eighth day of pouch development each rat received 2 intraperitoneal injections of 50 μc of carboxyl-labeled glycine§ 24 and 15 hours before the time of sacrifice. On the ninth day the animals were decapitated, and the croton oil pouches were cut open and placed immediately into 0.9% NaCl to remove croton oil and hemorrhagic inflammatory exudate. After wet weights were obtained, the tissue was cut into 4 to 5 mm pieces and placed into cold 0.9% NaCl until time of pulverization. Three x 6 cm sections of skin adjacent to the pouch were also taken. The segments were laid upon a flat surface, excess fat was removed, and the dermis was scraped off with a scalpel. The dermis was weighed, cut into pieces, and placed into cold NaCl solution. Tissues were frozen in liquid air and pulverized by means of a stainless steel mortar and pestle. The powdered tissue was then suspended in 10 ml of cold 4% perchloric acid to extract free amino acids, nucleotides, and other acid soluble substances. The suspension was centrifuged and the supernate saved for radioactivity measurements. The protein residues were resuspended twice in 10 ml portions of cold 4% perchloric acid, centrifuged, and the supernate added to the acid soluble fraction. In order to be certain that the residues were free of acid soluble substances, they were rinsed twice more with cold perchloric acid and the washings discarded. Lipids were extracted from the residues with 10 ml portions of 80% ethanol, 95% ethanol, two 10 ml portions of absolute ethanol, and two 10 ml portions of ether. The lipid-containing ethanol and ether extracts were pooled and radioactivity measurements made on these fractions. The ether extracted protein residues were allowed to dry in a desiccator overnight. A measured amount of the dried residue (3 to 5 mg) was then plated on tared aluminum planchets using 2 or 3 drops of water to distribute the residue evenly. The samples were counted in a Geiger-Mueller windowless gas flow counter. Radioactivity was expressed as counts per minute per milligram of protein. A correction for self-absorption and counting efficiency was made. Extraction and hydrolysis of nucleic acids was accomplished by treating the protein residues with 4% perchloric acid at 90°C for 30 minutes. Purine bases present in the perchloric acid extract were eluted from a Dowex 50 column with 6 N HCl according to the method of LePage(6). Guanine and adenine were separated by descending paper chromatography using a solvent system of tertiary butyl alcohol and constant boiling HCl as described by Markham and Smith(7). After locating the bases on the chromatographic strips with the aid of ultraviolet light, guanine and adenine were eluted with 1 N HCl. The eluate, which was collected in small beakers, was then evaporated to dryness with a stream of air, plated in .01 N HCl, and the radioactivity counted. In order to calculate the concentration of purine on the planchets, optical densities in 0.1 N HCl were

† Mono Beta-aminopropionitrile Fumarate was obtained from Abbott Laboratories, North Chicago, Ill.

‡ Purina Lab Chow.

§ Glycine-1-C^{14} was purchased from Tracerlab, Inc., allocated by U.S.A.E.C.



Formalin fixed sections of croton oil pouches removed from rats after 3, 6, 9 and 15 days. Before fixation fibrin and inflammatory cells were removed from the inner aspect of the capsule, which is shown at top of each photomicrograph. Sections were stained with hematoxylin and eosin. × 350.

FIG. 1. Three-day-old croton oil pouch. There is a mixture of fibroblasts and leukocytes in the capsule. Stroma is poorly developed.

FIG. 2. In the 6-day-old capsule immature fibroblasts with round or oval nuclei predominate. Stroma is more dense than at 3 days. Several cystic spaces can be seen in the capsule.

TABLE I. Radioactive Glycine Uptake into Proteins of Rat Croton Oil Pouches and Dermis.

	Wt at start of exp., g	Net gain, g	Wet wt of pouch, mg	Protein residue of pouch	Protein residue of dermis
				cpm/mg protein	
Exp. 1					
Controls	84.5	36.0	1220	5760	1905
	91.8	28.5	1310	5130	1610
	97.6	27.7	560	3430	1685
Test	91.4	23.5	520	3900	1559
	92.5	25.5	660	3790	1392
	89.1	22.4	600	3390	1416
Exp. 2					
Controls	86.0	13.5	790	4920	2280
	85.1	20.4	720	4420	1760
	79.6	16.9	670	3060	1500
Test	84.0	18.5	500	3590	1744
	80.2	15.8	400	2750	2100
	83.5	17.5	350	2580	918

determined in a Beckman DU spectrophotometer at 260 and 250 millimicrons for adenine and guanine, respectively. The concentrations were then calculated according to the molar extinction coefficients used by Markham and Smith(8). Radioactivity was expressed as counts per minute per micromole of adenine or guanine.

Five mg aliquots of the protein residues from croton oil pouches were hydrolyzed for 15 hours at 100°C with 4 N HCl. Following hydrolysis the samples were evaporated to dryness at 40°C under a stream of air and redissolved in 0.5 ml of water. Two 25λ portions were applied in 4 cm adjacent bands on Whatman No. 1 filter paper buffered with a mixture of 1 M HAc and 0.6 M HCOOH at pH 2.3. After 2 hours' electrophoretic migration at 14000 volts the filter paper strip was cut lengthwise to separate the duplicate samples, and amino acids were located by dipping one of the strips in 0.2% ninhydrin in acetone. The amino acids of the duplicate strip then were eluted from segments which corresponded to the ninhydrin bands of the developed strip. The eluates were plated and the radioactivity counted.

Results. Microscopic studies of the croton oil pouch. A preliminary time study was carried out to establish the phase of pouch development at which there are maximum numbers of young fibroblasts. Croton oil pouches were removed from 2 animals at 3, 6, 9, 12, and 15 days. Pieces of each pouch were fixed in 10% formalin, embedded, sectioned, and stained with hematoxylin-eosin. Microscopic findings demonstrated that the capsules of 3-day pouches contain many inflammatory cells (Fig. 1). By the sixth day almost all inflammatory cells have disappeared. The capsule is principally composed of immature fibroblasts containing large, round vesiculated nuclei. Minimal numbers of collagen fibers are present at 6 days (Fig. 2). At 9 days the capsule contains more collagen. The fibroblasts now contain round and fusiform nuclei (Fig. 3). On the twelfth day of development fusiform fibroblasts and small oval nuclei are conspicuous. The ratio of collagen to cellular elements is markedly increased. By the fifteenth day the capsule is composed mostly of collagen. Mature fibroblasts with fusiform nuclei now constitute over 75% of all fibroblasts (Fig. 4). Feeding BAPN to

FIG. 3. At 9 days fibroblasts with fusiform nuclei are conspicuous for the first time. Immature fibroblasts with round nuclei are more numerous near internal border of capsule. Many fibroblasts oriented parallel to the lumen. A capillary is evident in this segment of the pouch wall. Stroma is still poorly developed although collagen fibers are apparent.

FIG. 4. Fifteen-day-old capsule has a well defined collagen stroma. Most of fibroblasts are of adult type with fusiform nuclei. Few immature fibroblasts with round nuclei can still be seen at inner border.

rats delays maturation of the fibroblasts and delays collagen synthesis. Since maximum numbers of young fibroblasts are present at 7 to 9 days, the 8-day-old pouch was chosen as a source of connective tissue to study metabolic behavior of radioactive glycine as influenced by BAPN.

In vivo studies using radioactive glycine. The results of duplicate experiments 1 and 2 indicate that BAPN exerts a significant effect on rate of incorporation of radioactive glycine into proteins of granuloma and dermal tissues of rats. The data in Table I demonstrate from 23 to 28% less uptake of glycine in capsules of croton oil pouches from BAPN treated animals. Somewhat less affected by BAPN is rate of glycine incorporation into the dermal protein; those from BAPN fed animals have approximately 15% less activity than controls. The protein residues from both croton oil pouch and dermis show high specific activities. As one might expect, the uptake of radioactive glycine into the proteins of rapidly developing connective tissue, such as the croton oil pouch, is almost 3 times as great as in the more slowly metabolizing dermis.

Electrophoresis of the protein hydrolysates separates the following amino acids: aspartic acid, glutamic acid, methionine, threonine, serine, alanine, and glycine. Only in the serine and glycine bands is there significant radioactivity. The results show that, even though there is less total radioactivity in the protein residues from BAPN fed rats as compared to controls, the percentage of the radioactivity accounted for as glycine and serine in both test and control pouches is the same. Fifty-three % of the radioactivity is found in the glycine band in both the control and treated croton oil pouches. Thirty-six % of the radioactivity is accounted for by serine.

The incorporation of radioactive glycine into the purine bases of nucleic acids is given in Table II. The data of duplicate experiments demonstrate that the specific activities of guanine and adenine are essentially the same in both the control and BAPN treated tissues. On the basis of these experiments it appears, then, that BAPN does not affect the rate of incorporation of radioactive glycine into the purine bases of nucleic acids in croton oil pouches and dermis of the rat, whereas significant differences in the incorporation into protein are evident. The fact that animals continue to grow despite administration of BAPN suggests that the effect is a relatively local one, showing selective specificity in producing extracellular protein.

TABLE II. Radioactive Glycine Uptake into Nucleic Acids of Rat Croton Oil Pouches and Dermis.

	Adenine of pouch	Adenine of dermis	Guanine of pouch	Guanine of dermis
	cpm/μM adenine		cpm/μM guanine	
Exp. 1				
Controls	1753	1470	2750	2100
	1390	1525	2040	2290
	1230	1585	1800	2340
Test	1305	1493	1900	2250
	1420	1583	2160	2120
	1300	1680	1830	2030
Exp. 2				
Controls	1756	1583	2990	3030
	2000	1454	2680	2560
	1430	1650	1985	2660
Test	1550	1895	2120	3230
	1353	2700	1530	4150
	1370	1475	2010	2150

A preliminary analysis of the acid soluble fraction indicates only a small uptake of glycine into acid soluble substances. Measurements of radioactivity in the lipid fraction indicate that almost no labeled material is incorporated into this fraction.

Discussion. Our experimental data are compatible with the findings of Mielke, *et al.* (4) who demonstrated that collagen synthesis is in some way inhibited by BAPN. Their experiments indicated differences in the chemical composition and weights of croton oil pouches from animals fed BAPN as compared with controls. Test pouches contained from 20 to 25% less hydroxyproline in protein residues prepared similarly to those in the present investigation, the hydroxyproline determinations indicating amounts of collagen present. These workers also found that pouches from BAPN treated animals weighed only half as much as control pouches; the present work indicated similar results.

Utilizing glycine-1-C^{14} we have shown that BAPN significantly decreases amino acid incorporation into the protein residues of croton

oil pouches and dermis, but incorporation into the purines of nucleic acids from either of these tissues is unaffected. The data here reported do show some variation in response, which might possibly be accounted for by individual animal variations. It is interesting to note, however, that in croton oil pouch protein residues, the activity of the tissue is directly proportional to the weight of the pouch. Where the weight is high, as in the control tissues, specific activity is also high. One control tissue which, for some unknown reason, did not respond to the croton oil injection, showed lower radioactive glycine incorporation, similar to the incorporation found in test pouches.

To evaluate its specific role in connective tissue metabolism, we have attempted to determine whether BAPN acts primarily on regulation of cellular uptake or utilization of amino acids in collagen synthesis. Since glycine uptake into acid insoluble purines is unaltered by feeding BAPN, it would appear that BAPN does not exert its effect at the level of nucleic acid synthesis in the cellular component of the connective tissue. On the other hand, the observed lowering of glycine incorporation into the protein residue is compatible with earlier findings indicating an inhibitory action of BAPN on the synthesis of extracellular collagen by these same cells.

Summary. 1. An investigation of the influence of BAPN on incorporation of glycine-1-C^{14} into proteins and nucleic acids of rat croton oil pouches and dermis was undertaken. 2. Incorporation of glycine into protein residues of fibrous tissue from croton oil pouches was decreased about 25% in animals pretreated with BAPN. In protein residues of dermis the rate of incorporation of glycine was also decreased, but to a much lesser degree, about 15%. 3. Studies of the nucleic acid fraction indicate that no change occurs in glycine uptake in fibrous tissue of dermis or croton oil pouches from BAPN treated animals as compared to controls. 4. The results of these experiments suggest that BAPN primarily affects amino acid incorporation into collagen or ground substance rather than the cellular portion of connective tissue.

1. Dasler, W., *Fed. Proc.*, 1954, v13, 519.
2. Ponseti, I. V., and Shepard, R. S., *ibid.*, 1954, v13, 473.
3. Bachhuber, T. E., Lalich, J. J., Angevine, D. M., Schilling, E. D., and Strong, F. M., PROC. SOC. EXP. BIOL. AND MED., 1955, v89, 294.
4. Mielke, J. E., Lalich, J. J., and Angevine, D. M., *ibid.*, 1957, v94, 673.
5. Selye, H., *ibid.*, 1953, v82, 328.
6. LePage, G. A., *Cancer Res.*, 1953, v13, 178.
7. Markham, R., and Smitth, J. D., *Biochem. J.*, 1950, v46, 509.
8. ———, *ibid.*, 1951, v49, 401.

Received July 22, 1957. P.S.E.B.M., 1957, v96.

Thresholds for Pain and Convulsions in the Guinea Pig Following Massive Whole-body Irradiation. (23473)

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The guinea pig shows an abrupt shortening of survival time and a striking change in mode of death(1) when a single whole-body radiation dose exceeds 6,000 r (6 Kr). Below 6 Kr the clinical picture is essentially that of depression, while above 6 Kr excitation, with frequent convulsions, is regularly seen. Pentobarbital sodium, given prior to radiation, prevents the clinical signs of excitation(2) and lengthens survival time. The observable neuropathology(3) appears insufficient to account for the early death and this suggested the present study on the functional state of some accessible neurological systems.

Previous studies(4) have shown only small and subtle effects on the electroencephalogram at doses below 6 Kr and paroxysmal seizure waves at higher doses. Changes in the elec-

TABLE I. Convulsive Thresholds.

Radiation (Kr)	Milliamperes avg* at various times (hr)							Avg survival (hr)
	Pre r	*2–6*	*24–29*	*48*	*72*	*144*	*168*	
5	20	20	19	23	22	25	23	158
		.2	*1*	*6*				
10	22	15	4	2				12
		28	*49*	*75*				
10†	19	17	22	18				121

* Each value is avg of 2 to 4 animals.
† Pentobarbital sodium (35 mg/kg) i.p. preceded radiation exposure.

trocardiogram are small except when the animal is moribund. One of the more prominent features of the high-dose syndrome is the hypothermia which appears early and may persist until death. Gerstner *et al.*(5) reported a rise in pinna reflex threshold 3 hours after 8 Kr to the head. Twenty-four hours after irradiation the pinna reflex was abolished. We have obtained essentially similar results with the pinna reflex but have experienced difficulty in obtaining consistent responses. The present report concerns convulsive thresholds as elicited by electric shock and "pain threshold" determined by reaction to thermal radiation. Sharp end points and reproducible responses can be obtained with both of these reactions.

Methods. Adult male guinea pigs (800-1200 g) from the NIH hybrid stock were used. All radiations were delivered with a Van de Graaff generator operating at 2.5 Mev, TSD 75 or 100 cm, dose rate 500-2,000 r/min., HVL 10 mm lead. All doses were administered by an integrating ionization chamber circuit, calibrated before each run against a thimble chamber. Electric shocks were delivered through electrodes clipped to the ears after washing with acetone and vigorously applying conducting electrode paste. Stimuli of 3 sec. duration were delivered by a motor driven switch. The stimulus consisted of a 60 cycle alternating current supplied by a transformer through a high series resistance which reduced the effect of varying electrode resistance. Current was measured by the voltage drop across a standard resistor. Convulsive threshold was taken as that current required to produce a convulsion with both tonic and clonic components. Current was varied in steps of about 2.5 milliamperes and no attempt was made to obtain readings closer than this. Below-threshold stimuli were spaced at least 15 minutes apart to prevent summation. "Pain threshold" measurements used essentially the procedure(6) developed for the dog. Radiation from a 750 watt projection bulb was focussed onto the shaved, blackened skin of the back. Three-second stimuli were repeated at 30-second intervals. Threshold was taken as the watts input to the lamp needed to elicit a characteristic muscular twitch(7). This may not be a response to pain but it is at least closely related and involves a large segment of the nervous system.

Results. Table I shows the results of convulsive threshold measurements in animals receiving 5 Kr, 10 Kr, and 10 Kr preceded by 35 mg/kg pentobarbital sodium, i.p. In the latter group post-radiation measurements were not made until several hours after each animal had returned to an apparently normal state of alertness.

The 10 Kr data shown in Table I were taken when no spontaneous convulsions were occurring. Handling incidental to electrode placement did not elicit convulsions but after 10 Kr these were always produced at the lowest current value tested. From this fact, and the length and severity of the induced seizures it seems probable that the 10 Kr post-r values may be somewhat lower than those given. It did not seem worthwhile to establish more exact values.

As Table I shows, the drop in threshold at 10 Kr occurs rapidly but is not coincident with absorption of the radiation. Threshold values were nearly normal in 2 animals tested 12 minutes after the mid-point of a 10 Kr, 10 min irradiation, but at 1 hour each unmedicated animal had a low value.

Table II gives the results of the pain

TABLE II. Pain Thresholds.

Radiation (Kr)	Watts avg* at various times (hr)								Avg survival (hr)
	Prev	*1*	*24*	*96*	*120*	*144*			
5	176	181	179	173	186	196			147
		.5	*5*						
10	169	170	191						12
		.1	*2*	*24*	*−48*	*+48*	*139*	*163*	
5 + 5,† 48 hr later	177	194	169	158	184	233	244	220	156

* Each value is avg of 4 animals except at 139, 144 and 163 hr where only 3, 3, and 1 animals, respectively, were surviving.

† Time measured from the first of 2 exposures.

threshold measurements.

Pain-threshold data from animals receiving a single dose of 5 Kr show no significant trends although there was a tendency toward somewhat higher values a few hours before death. Data obtained from animals receiving 10 Kr and pentobarbital were similar to those from 5 Kr animals and have been omitted from Table II. The 10 Kr data at 5 hours are presented as found but are of dubious value because almost continuous muscle twitching obscured the sharp end points usually obtained.

Divided dose experiments have been done with intervals between irradiations ranging from 2 to 48 hours. The large, prompt increase in threshold shown in Table II was seen only at the longest interval. A small prompt increase was seen when the doses were spaced 19 hours apart but at shorter intervals no prompt effect of the second dose was evident.

Discussion. The convulsive threshold data confirm what would have been inferred from clinical observations, and emphasize the differences existing between guinea pigs receiving less than, or more than, 6 Kr whole-body radiation. The suppression of clinical signs of convulsions by pentobarbital undoubtedly results from its ability to prevent the sharp drop in threshold usually seen after 6 Kr or more. At 5 Kr there is no significant trend in the pain threshold data although the values tend to rise as the animal becomes moribund. The abrupt, prompt rise in threshold observed after a second 5 Kr dose appears much like a sensitivity reaction. The high values observed with the divided dose schedule are not approached at any time with a single 5 Kr dose. Observations following 10 Kr are difficult, but it appears that here also pain threshold values are lower than those seen with the 2 doses spaced 48 hours apart.

The relative constancy of the pain threshold response indicates that function is relatively unimpaired in a large portion of the nervous system. The responses obtained may not represent true pain thresholds but certainly afferent and efferent fibers are involved and thalamic integration and cortical localization of response seems to be required. The hypothalamic areas probably responsible for the observed hypothermia, and the cerebellar areas showing pyknotic cells are presumably not involved in the reactions studied here.

Summary. Pain thresholds were tested using the thermal radiation from a projection bulb as a stimulus, and convulsive thresholds to electric shock were determined with ear electrodes. Radiation doses of 5,000 r and 10,000 r were used to bracket the critical 6,000 r dose, above which signs of nervous system injury predominate. Pain thresholds remained relatively constant at each radiation dose until the animals became moribund. Convulsive thresholds were relatively constant at a dose of 5,000 r but dropped sharply following 10,000 r. Pentobarbital given prior to 10,000 r prevented the drop in convulsive threshold even after the obvious drug effects had disappeared. The essential constancy of the pain threshold response indicates that function is relatively unimpaired in a large portion of the nervous system.

1. Brace, K. C., Andrews, H. L., and Thompson, E. C., *Am. J. Physiol.,* 1954, v179, 386.

2. Andrews, H. L., and Brace, K. C., *ibid.,* 1956, v187, 378.

3. Alvord, E. C., and Brace, K. C., *J. Neuropath. and Exp. Neurol.*, 1957, v16, 3.

4. Andrews, H. L., and Brace, K. C., *Am. J. Physiol.*, 1953, v175, 138.

5. Gerstner, H. B., Konecci, E. B., and Taylor, W. F., *NP* (4856) *Rep.* 8, Sept. 1953.

6. Andrews, H. L., and Workman, W., *J. Pharmacol. and Exp. Ther.*, 1941, v73, 99.

7. Winder, C. V., *Yale J. Biol. and Med.*, 1947, v19, 289.

Received July 22, 1957. P.S.E.B.M., 1957, v96.

Neonatal Immunity. I. Disparity in Maternal-Infant Poliovirus Antibody.* (23474)

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The studies here reported concern the titers of poliovirus antibodies to the 3 serotypes in the serum of mothers and their newborn infants. It represents part of a study on the local formation and transfer of antibody *in utero*. Since we are dealing exclusively with infants at the moment of birth, the possible role of colostrum will not be discussed nor will the uncertain role of the placenta in production of antibodies. The subject of active and passive immunity of the infant has been reviewed by Edsall(1). Previous reviews include one on placental permeability by Doerr (2). One of the earliest observations of antibody transfer in the human from mother to infant concerned that of diphtheria in 1895 (3). Later studies demonstrated transmission of tetanus antibodies(4). More recently, however, the concern has been with the quantitative aspects of maternal transmission of antibody and, therefore, neonatal immunity.

Although one might expect all antibodies to behave similarly with respect to maternal-infant transmission, this does not appear to be the case. Reagins reportedly do not appear in the umbilical serum whereas blocking antibodies do(5). Viruses may differ among themselves in this respect, thus in Japanese B encephalitis(6) and mumps(7) no disparities have been observed whereas they have been found in vaccinia(8). Employing untyped poliovirus in qualitative tests in monkeys, Aycock and Kramer reported neutralizing antibody in 10 of 12 matched maternal-cord serums tested(9). Among the 3 serotypes of polioviruses we have observed disparities in titer most marked in Type 3. Rather than stress the anticipated agreements we have chosen to consider the possible meaning of the observed disparities among the 3 serotypes.

Methods. A series of 48 mothers' and corresponding umbilical cord bloods were collected aseptically generally at term; this includes 4 prematures and 3 sets of twins. The immunization record of each mother in regard to poliomyelitis was recorded but not known at the time of antibody titration. Mothers were of various ages, color, and previous obstetrical history who were seen in pre-natal clinics and delivered at a city hospital. The antibody titrations were performed *in vitro* by a modification of the technic originally published by Salk, Youngner and Ward(10). Monkey kidney cells were obtained by seeding bottles with trypsinized monkey kidney tissue. After 1 week the cells were harvested by use of versene and used in a suspended cell neutralization test using 2% calf serum, 0.5% lactalbumin hydrolysate in Hanks' BBS. Twenty thousand cells were used/tube. The matched maternal and umbilical cord serums were always titrated simultaneously, dilutions of both being made by the same worker. For antibody titrations versus each virus type, 100 TCD_{50} of poliovirus either type 1 (Mahoney), type 2 (MEF_1), or type 3 (Saukett)

* This investigation was supported by research grant from Natl. Inst. of Allergy and Infect. Dis., U.S.P.H.S.

TABLE I. Antibody Titer Distribution for Polioviruses in Maternal and Corresponding Cord Serums. No disparities.

Age	P/G*	Type 1 MB†	Type 1 CB†	Type 2 MB	Type 2 CB	Type 3 MB	Type 3 CB
No record of poliomyelitis vaccination							
16	1/ 1	1024‡	1024	4096	4096	32	32
17	1/ 1	512	512	512	1024	256	256
18	1/ 1	64	64	128	128	128	128
20	2/ 2	32	16	128	128	128	64
21	1/ 1	512	512	512	512	512	512
22	2/ 2	64	64	2048	2048	256	256
24	4/ 4	512	256	128	128	128	128
25	4/ 5	<4	<4	512	512	64	64
26	—	64	128	256	256	32	32
29	6/ 6	32	32	1024	512	64	64
35	3/ 3	128	128	256	256	64	64
39	11/14	32	16	64	64	16	16
42	4/ 4	512	512	2048	2048	256	256
—	8/ 9	128	128	512	512	128	128
1 ml of poliomyelitis vaccine							
15	1/ 1	512	512	512	512	512	512
19	1/ 1	512	512	16	16	256	256
22	3/ 4	2048	2048	2048	2048	256	256
27	—	256	256	512	256	128	128
2 ml of poliomyelitis vaccine							
14	1/ 1	1024	1024	1024	1024	64	64
16	1/ 1	<4	<4	4	4	<4	<4
19	1/ 1	1024	2048	512	512	512	512
19	3/ 4	32	32	256	256	8	8
20	2/ 2	32	32	1024	512	<4	<8
§21	3/ 3	512	512	2048	2048	2048	2048
22	3/ 3	1024	1024	4096	2048	512	256
23	4/ 5	512	256	2048	2048	2048	2048
25	3/ 3	64	128	256	128	256	128
25	4/ 4	1024	512	—	—	—	—
26	6/ 6	2048	1024	512	512	2048	2048
28	6/ 6	128	128	128	128	1024	1024
29	7/ 7	128	128	512	512	512	256
29	6/ 8	16	8	512	1024	128	128
30	11/11	32	32	128	64	256	128
38	5/ 5	256	256	512	256	256	256

* P/G = Parity-gravid status.
† MB = Maternal blood; CB = Cord blood.
‡ Titer expressed as reciprocal of last serum dilution neutralizing virus.
§ Double ovum twins.

was admixed with doubling dilutions of serum using 2 or 3 tubes per serum dilution per type of virus. After incubation at room temperature for 1 hour, cells were added, the tubes were stoppered and incubated at 36°C. Endpoints were recorded as the last dilution which on microscopic examination at 5 to 7 days did not show degeneration or cytopathogenicity. In instances where large differences were found between mother serum and the corresponding cord serum the titration was repeated. For the purposes of this study a deviation of 1 twofold dilution was considered to be within the realm of experimental error; differences of 4-fold were considered noteworthy especially when observed upon repetition. Standard monkey antisera of the 3 serotypes† were titrated with each test. The type specific viruses were also titrated for each test.

Results. In the accompanying Tables I and II are tabulated serum neutralizing antibody titers of the matched maternal and cord sera listed in the order of the patient's poliomyelitis vaccination status and the maternal age. Table I enumerates those matched pairs of sera in which no significant disparities in titer (as described under methods) appeared. Table II enumerates those instances in which differences beyond the realm of experimental error were observed. The disparities do not appear to be related to maternal age, parity or poliomyelitis immunity status of the mother nor to the sex, color or birthweight of the newborn.

Two points may be seen in Tables II and III. In all instances where differences are noted the maternal serum has a higher titer than the corresponding cord serum. Secondly, the largest number of disparities is to be found in the antibody to the type 3 poliovirus with differences up to 64-fold. Type 2 antibody disparities appear less frequently. In only one instance it is the lone disparity (mother MC). Otherwise type 2 disparities are accompanied by type 3 differences and types 1 and 3 differences (Table III). In 1 instance (mother GS) the difference between maternal and cord serum is 64-fold for type 2 while only 4-fold for type 3. In 2 cases type 1 antibodies are higher in the maternal circulation than in the cord serum in the absence of antibody differences between these sera to the other poliovirus types. In both of these cases the disparity is only 4-fold. The other instances of a type 1 antibody disparity are in the presence of type 2 and 3 differences which in one instance (mother MB) are much greater for types 2 and 3 (64-fold) than for type 1 (16-fold).

One case is of special interest and may have

† Prepared by Dr. Herbert A. Wenner, University of Kansas.

TABLE II. Antibody Titers *vs* Polioviruses in Maternal and Corresponding Cord Serums. Disparities.

Mother	Age	P/G	Type 1		Type 2		Type 3	
			MB	CB	MB	CB	MB	CB
			No record of poliomyelitis vaccination					
CB	14	1/ 1	128	128	<4	<4	16	4
GS	16	1/ 1	128	64	256	<4	128	32
MS*	32	11/12	256	64	32	8	16	<4
			1 ml of poliomyelitis vaccine					
AF	19	2/ 2	512	512	1024	512	2048	64
MB†	26	7/ 7	1024	64	1024	16	1024	16
AN‡	28	5/ 5	256	256	512	512	16	<2
DL	29	4/ 4	128	32	2048	1024	1024	1024
			2 ml of poliomyelitis vaccine					
MP	18	5/ 5	256	128	1024	256	512	128
RG	22	4/ 4	512	512	128	128	4	0
JH	22	2/ 2	256	256	512	512	128	32
CN	27	8/ 8	32	16	64	8	128	4
EB	31	6/ 6	256	256	256	64	256	32
MC	33	7/ 7	64	64	512	128	128	128
			3 ml of poliomyelitis vaccine					
MG§	30	5/ 5	512	128	1024	1024	512	512

For abbreviations see footnotes Table I.

* 30 wk gestation. † 1 ml vaccine 1 wk before delivery. ‡ Double-ovum twins.
§ Single-ovum twins.

clinical significance. In Table II it can be seen that mother MB was given 1 ml of poliomyelitis vaccine 1 week prior to delivery. The significance of the striking differences observed between maternal and cord serum will be covered in the discussion.

In Table IV are the results obtained with sera from 3 sets of twins, 2 sets of which were heterozygous (mothers AN and AW) and the last uniovular (mother MG). In the AW twins antibody titers are approximately the same in all 3 specimens against the 3 poliovirus types. In the AN family types 1 and 2 antibodies are in equal titer for all 3 specimens. For type 3 poliovirus, however, the maternal serum antibody is 8-fold higher than the cord serums, both of which are the same. In the uniovular twins (mother MG) the type 1 antibodies are of 4-fold lower titer in the cord serum of each of the twins than in the maternal serum. Antibodies to the other poliovirus types are the same for all 3 specimens.

Discussion. The reason for the observed disparities in titer between maternal and umbilical cord serum is not known, nor for the disparity to be more frequent (Table III) for types 3, 2, and 1 polio antibody, in that order. The possible role of a bio-physical explanation is suggested by the work of Timasheff(11) who utilized electrophoresis convection to partition the antibodies of type 1 and 2 poliovirus; no observations were reported for the type 3. He observed human type 2 antibody in highest concentration (37-fold) in a slow-

TABLE III. Tabulation of Observed Titer Disparities by Serotype of Virus.

Mother	Type 1	Type 2	Type 3
DL	4*†		
MG‡	4		
MC		4	
RG			>4
AF			32
JH			4
CB			4
AN‡			>8
MP		4	4
EB		4	8
CN		8	32
GS		64	4
MB§	16	64	64
MS	4	4	>4

* In all instances maternal higher than corresponding cord serum.
† Indicates fold difference in titer between maternal and cord serums.
‡ Twins—both twins had same titers.
§ Received 1 ml poliomyelitis vaccine 1 wk prior to delivery.

TABLE IV. Poliomyelitis Antibody Titers of Maternal and Cord Serums of Twins.

Mother	Age	P/G*	ml polio vaccine	Spec. source	Sex of newborn	Type of twin	Poliovirus antibody Type 1	Type 2	Type 3
AN	28	5/5	1	Mother			256	512	16
				Twin 1	♂	Double ovum	256	512	<2
				" 2	♀		128	512	<2
AW	21	3/3	2	Mother			512	2048	2048
				Twin 1	♂	*Idem*	512	2048	2048
				" 2	♀		512	1024	2048
MG	30	5/5	3	Mother			256	1024	256
				Twin 1	♀	Single ovum	64	1024	256
				" 2	♀		64	1024	256

* P/G = Parity-gravid status.

moving gamma globulin fraction T 1. T 2 fraction of slightly greater mobility revealed a concentration of 17x while the T 3 fraction revealed antibody only 5x as active as the original serum. On the other hand the type 1 antibodies revealed maximum concentration (43x) in the T 4 gamma globulin fraction of intermediate mobility. In the monkey hyperimmunized with type 2 live attenuated virus, specific antibodies appear principally in a slow-migrating β globulin fraction rather than in the gamma globulin component(11).

It would appear that the configuration and mobility of the several specific types of antibodies varies in complexity. Our data suggest that Type 1 antibody was most easily transferred from mother to offspring; the fact that the Type 1 poliovirus is the poorest antigen of the three, may be related to its yielding antibody particles which are smaller and less complex, hence easily transferred. Such physical factors might explain failure of antibody (hemagglutinins) to the pathogenic *E. coli* being transferred to the human newborn while antibody to the non-pathogenic *E. coli* are transferred(12).

How and when do the various antibodies find their way from the maternal circulation to that of the newborn? Patient MB (Table III) injected with 1 ml of poliomyelitis vaccine (Salk type) had serum titers of 1:1024 against all 3 types of virus when delivered a week later. This apparent booster response in her did not affect the infant's titers, whose cord serum revealed 1:64, 1:16 and 1:16 against types 1, 2, and 3 viruses respectively. Brambell's work(13) raises the question of several independent mechanisms being involved in the transfer of antibody from mother to offspring.

It would appear that further investigation regarding human neonatal immunity will be required in order to clarify when and by which mechanisms the transfer of antibody occurs.

Summary. Neutralizing antibody titrations for the 3 poliovirus serotypes performed on maternal and umbilical cord bloods at 48 deliveries revealed disparities of 4 to 64-fold in 14 individuals, the maternal titer always being higher. Double ovum twins had identical titers with each other, though not necessarily with their mother. The least frequent disparity was for the Type 1 antibody. The possible role of physical factors affecting the transfer of the different serotype antibody molecules is discussed.

1. Edsall, G., *Ann. N. Y. Acad. Sci.,* 1956, v66, 32.
2. Doerr, R., *Schweiz. Med. Wochschr.,* 1941, v71, 1253.
3. Fischl, R., and von Wundscheim, *Z. Heilk.,* 1895, v16, 429.
4. Polano, O., *Z. Geburtshülfe U. Gynakol.,* 1904, v53, 456.
5. Sherman, W. B., Hampton, S. F., and Cooke, R. A., *J. Exp. Med.,* 1940, v72, 611.
6. Hale, J. H., and Lee, L. H., *J. Path. and Bact.,* 1954, v68, 631.
7. Florman, A. L., Shick, B., and Scalettar, E., PROC. SOC. EXP. BIOL. AND MED., 1951, v78, 126.
8. Kempe, C. H., and Benenson, A. S., *J. Pediat.,* 1953, v42, 525.
9. Aycock, W. L., and Kramer, S. D., *J. Exp. Med.,* 1930, v52, 457.
10. Salk, J. E., Youngner, J. S., and Ward, E. N.,

Am. J. Hyg., 1954, v60, 214.

11. Timasheff, S. N., Moyer, A., Brown, R., and Kirkwood, J. G., *Proc. Nat. Acad. Sci.*, 1956, v42, 228.

12. Neter, E., Westphal, O., Lüderitz, O., and Gorzynski, E. A., *Proc. N. Y. Acad. Sci.*, 1956, v66, 141.

13. Brambell, F. W. R., Halliday, R., Brierley, J., Hemmings, W. A., *Lancet*, 1954, v1, 964.

Received July 22, 1957. P.S.E.B.M., 1957, v96.

Effect of Aureomycin on Hepatic Utilization of Labeled Body Fat in Rats.* (23475)

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Rats fed Aureomycin and given a tracer dose of $CH_3C^{14}OONa$ orally or parenterally 24 hours later have more C^{14} incorporated in hepatic fatty acids than control rats not treated with the antibiotic(1). In order to investigate the mechanism responsible for the increased radioactivity, we have studied the effect of administration of Aureomycin on mobilization of labeled body fat to the liver and on its subsequent accumulation in this organ. As part of this investigation we have made a 4-day balance study of orally administered C^{14}-tripalmitin in normal rats.

Materials and methods. Adult, male Sprague-Dawley rats were paired by weight and kept on Purina Laboratory Chow *ad libitum*. In order to label the body fat, a daily dose of 25 mg of carboxyl-labeled C^{14}-tripalmitin, contained in 1 ml of olive oil, was fed by stomach tube to each rat for 4 days. During this period and the subsequent day food was allowed *ad libitum*. The radioactive tripalmitin was not fed on the fifth day so that disappearance of absorbed labeled fat from the blood stream would be complete. On the sixth day 5 mg of Aureomycin (Lederle) were given by stomach tube to a member of each pair of rats. After administration of antibiotic the rats were fasted 24 hours and killed by decapitation. Control rats were treated similarly except that water was given instead of Aureomycin. Feces excreted during the first 5 days were pooled for each rat. Fecal samples of the sixth day were combined with the contents of the gastrointestinal tract of each animal. Immediately after decapitation of the animals the organs and tissues to be analyzed were placed in alcoholic KOH. After digestion and saponification of the samples chemical and radioactivity determinations of total fatty acids were made by procedures described previously(2).

Results. The livers of rats treated with Aureomycin contained fatty acids in amounts similar to those in the control group. The specific activity and total activity of the hepatic fatty acids of both groups also were similar. These data are given in Table I in the form of an average and standard deviation for each of the 2 groups. Examination of the data on the basis of each pair of rats also proved that there was no consistent significant difference between experimental and control animals.

In a similar manner, analyses of fatty acids of other tissues revealed no significant differences in amount or radioactivity between the 2 groups. Carcass total fatty acids of antibiotic-treated animals contained 32.5% (S.D. = 3.9) of the absorbed activity; those of controls contained 31.3% (S.D. = 5.8). Results of analyses of intestinal fatty acids were more variable and ranged from 0.78 to 2.04% of the absorbed activity in the experimental group and from 0.81 to 1.60% in the control group.

A 4-day balance period in all rats before administration of antibiotic resulted in an average absorption value of 88.1% (S.D. = 3.2) of the tripalmitin fed.

* This paper is based on work performed partly under contract with U. S. Atomic Energy Com.

† Work done while on a medical student research fellowship from National Fn. for Infantile Paralysis.

TABLE I. Amount and Radioactivity of Hepatic Fatty Acids of Aureomycin-Treated and Control Rats.

	Experimental		Controls	
	Avg	Stand. dev.	Avg	Stand. dev.
Wt of liver (g)	7.45	.95	7.18	1.18
Total fatty acids				
% of wet wt of liver	3.75	.96	3.68	.50
Specific activity (cpm/mg fatty acid)	26.52	5.32	23.55	4.32
Total activity (total cpm/liver)	7130	1400	6120	1300
Total activity (% of total body counts × 100)	25.3	3.9	22.8	5.1

All except one pair of animals showed a net loss in weight, ranging from 13 to 27 g, during the experimental period. This was due largely to the 24-hour fasting period immediately prior to sacrifice.

Discussion. No excessive amount of fat had accumulated in the rats given Aureomycin and killed after a 24-hour fast. This problem is of interest in view of conflicting reports on effects of antibiotics on liver lipids (3-5). Aureomycin was observed to cause reversible fatty metamorphosis in the liver of man(6) and to be antilipotropic in rats over a period of 15 days(7), but it was reported by György *et al.*(8) to exert lipotropic action in rats maintained on choline-deficient diets for relatively long periods of time. Since our animals received only one dose of Aureomycin and lived only 24 hours after administration of the drug, gross changes in liver lipids would not be expected. However, experiments reported previously(1) demonstrated increased amounts of C^{14} in liver fatty acids of rats given Aureomycin and fed a tracer dose of C^{14}-sodium acetate 24 hours later. These findings cannot be explained in terms of increased mobilization to the liver or decreased utilization by the liver since, in the present experiments, similar C^{14} activity was found in hepatic fatty acids of both experimental and control rats, the body fat of which had been labeled previously with C^{14}-tripalmitin. That excessive mobilization from carcass stores of fat had not occurred is indicated also by the observation that carcass total fatty acids of both groups had similar amounts of C^{14} activity. Because of the large amount of C^{14} in the carcass compared to liver, it is possible that this measurement lacked the sensitivity required to detect a significant difference between the 2 groups. Our results are in accord with the hypothesis that the observed immediate effect of Aureomycin is on lipogenesis.

The 4-day fat balance study showed that from 7 to 19% of the activity fed was recovered in the feces and intestinal contents, indicating satisfactory absorption in the presence of a relatively large amount (1 ml) of oil administered. Our results agree with those of Hoagland and Snider(9) who observed that about 15% of the tripalmitin fed in 5 or 10% solution in olive oil was excreted. Values of absorption ranging from 57 to 92% for labeled triolein were reported by Bergström *et al.*(10) in 24-hour experiments in rats given one dose of the radioactive oil. Bergström and co-workers(11) also reported that stearic acid transesterified with corn oil was absorbed to the extent of 95% of the amount fed.

Of the radioactivity absorbed only from 20 to 40% was found in the combined tissues analyzed. Since these included the entire body except the head, 60 to 80% of the activity absorbed had disappeared from the body fat through metabolic conversion to CO_2 and other compounds.

Summary. Administration of 5 mg Aureomycin to rats, the body fat of which had been labeled previously with C^{14}-tripalmitin did not result in accumulation of excessive C^{14} activity in hepatic fatty acids. The amount and radioactivity of total fatty acids of carcass and of intestine were likewise similar in experimental and control animals. These findings are discussed in relation to the observation that Aureomycin causes an increase in

incorporation of C^{14}-acetate into hepatic fatty acids of rats. A 4-day metabolic balance study showed that from 81 to 93% of the fed tripalmitin was absorbed.

1. Coniglio, J. G., and Bell, E. J., *J. Biol. Chem.*, 1957, v226, 805.

2. Coniglio, J. G., McCormick, D. B., and Hudson, G. W., *Am. J. Physiol.*, 1956, v185, 577.

3. Lepper, M. H., Wolfe, C. K., Zimmerman, H. J., Caldwell, E. R., Jr., Spies, H. W., and Dowling, H. F., *A.M.A. Arch. Int. Med.*, 1951, v88, 271.

4. Sutherland, D. A., Mann, J. D., Giges, B., and Seligson, D., PROC. SOC. EXP. BIOL. AND MED., 1951, v77, 458.

5. Sborov, V. M., and Sutherland, D. A., *Gastroenterology*, 1951, v18, 598.

6. Yesner, R., and Kunkel, P., *Yale J. Biol. Med.*, 1951, v23, 299.

7. Abedi-Djourabtschi, M., and Hartmann, F., *Z. physiol. Chem.*, 1954, v298, 193.

8. György, P., Stokes, J., Jr., Goldblatt, H., and Popper, H., *J. Exp. Med.*, 1951, v93, 513.

9. Hoagland, R., and Snider, G. G., *J. Nutrition*, 1943, v26, 219.

10. Bergström, S., Blomstrand, R., and Borgström, B., *Biochem. J.*, 1954, v58, 600.

11. Bergström, S., Borgström, B., and Rottenberg, M., *Acta Physiol. Scand.*, 1951, v25, 120.

Received July 22, 1957. P.S.E.B.M., 1957, v96.

Studies on Variation in Virulence of Poliomyelitis Virus II. Role of Host Cells and Their Maintenance Medium. (23476)

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In developing strains of avirulent viruses which are satisfactory for immunization, it is important to find out whether there are any environmental factors which can affect the viral virulence. In a previous paper(1) a mouse avirulent variant, (now designated as LH-a), isolated from the mouse adapted Type III poliovirus was reported. On further study, this avirulent variant again yielded a virulent progeny, (designated as LH-v). These 2 variants served as testing tools and with them the effect of various factors on viral virulence was studied and the results are here reported.

Materials and methods. Tissue culture. Primary cultures of trypsinized monkey kidney cells were obtained from a commercial source,† or prepared according to the method described by Dulbecco and Vogt(2) and modified by Youngner(3). These cells were grown in 199 medium with 2% calf serum or in Melnick's medium(4) consisting of 0.5% lactalbumin hydrolysate in Hanks' balanced salt solution with 2% calf serum. After growing for about a week, when a complete sheet of cells was formed, the medium was poured off, the cells were washed and fresh medium was added. Such cultures 8 to 15 days old were ready for use in titration, neutralization or passage experiments.

Serial passage cultures of HeLa cells grown in Eagle's basal medium with 20% human serum were supplied commercially.†

All quantitation of the virus in the various harvests was carried out in monkey kidney tissue culture in roller tubes containing lactalbumin hydrolysate medium with 2% calf serum. Serial 1 log or half log dilutions of the virus were made in neutral Hanks' balanced salt solution and 5 or 6 tubes each containing 1.8 ml of medium, were inoculated with 0.2 ml of each of at least 4 dilutions. The tubes were incubated for 7 to 8 days. The tubes were observed microscopically every 2 or 3 days, and the ID_{50} was determined by the Reed and Muench method.

Media. Eagle's basal medium was prepared according to a recently modified formula(5,6). Medium 199 was purchased in

* U. S. Department of Health, Education, and Welfare, Public Health Service.

† Microbiological Associates, Inc., Bethesda, Md.

the concentrated form‡ or prepared by our own media room according to the procedure used by Youngner(3). Lactalbumin hydrolysate medium and Medium E (1 part of bovine plasma hydrolysate and 3 parts of Hank-Simms solution)(7) which contained no serum were prepared in this laboratory. Calf serum used in the preparation of all the media was supplied commercially.§ Every lot of the serum was shown to be free of antibodies or inhibitors against polioviruses before use. For comparison of different media or factors in the same experiment the same lot of serum was used.

Assay of effects of various factors on viral virulence. The effect of various factors including cells and media on virulence of the virus was tested in the following way: Consecutive passage of the testing virus, LH-a or LH-v was carried out in duplicate. Each set contained 5 to 8 tissue culture tubes, including controls without virus inoculation. The same lot of cells was used in each experiment and all the sets were transferred at the same time. From 4 to 10 or more passages were found necessary before loss or gain of virulence of the virus could be clearly demonstrated.

Selected monkey kidney tissue culture tubes were washed twice with the testing media after removal of the original media and then incubated for 24 to 72 hours in the testing media. The testing media were again changed immediately before virus inoculation. All the media were adjusted to the desired pH, 7.4-7.5, by adding diluted sodium bicarbonate solution unless mentioned otherwise. Then 0.2 ml of the undiluted virus in tissue culture fluid was inoculated into each tube containing 1.8 ml of the medium. After virus inoculation the pH of each tube was determined every day. Usually after incubation for 24 hours the pH dropped from 7.4-7.5 to 7.1-7.3 regardless of the medium used. Rarely did the pH drop below 7.0 and then it was brought up to 7.1-7.2.

The tissue culture fluid was harvested when the cellular degeneration became 4+, but never before the 4th day. The harvested virus was then stored and used as inoculum for the next passage. Selected specimens were tested in mice and titrated in tissue culture.

Virus. The LH-a virus as reported previously(1) was derived from the 72nd mouse cord passage of type III poliovirus and passed in the monkey kidney testicular tissue culture in Medium E for 15 passages followed by 4 passages in monkey kidney tissue culture in the same medium. The fluid was centrifuged and filtered through an ultra-fine sintered glass filter and stored in small amounts as stock virus at −50°C. The LH-v virus was prepared and stored in the same way except Medium 199 was used. The specificity of both viruses was repeatedly checked by neutralization tests with type III immune serum obtained from the National Foundation for Infantile Paralysis and also with immune rabbit serum prepared by us against the Saukett strain.

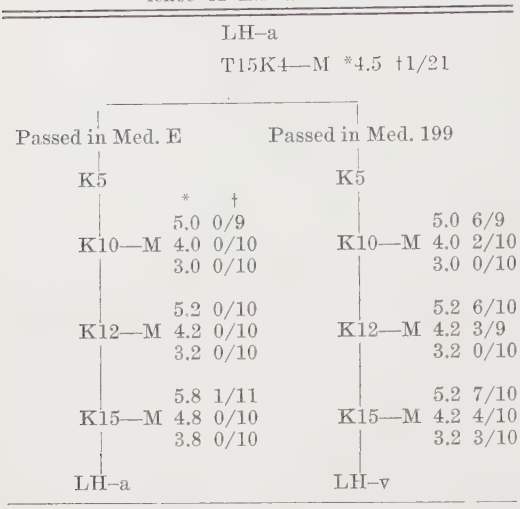
Mice. Assay of viral virulence was made by inoculating 0.02 ml of the undiluted and 10-fold dilutions of the virus in buffered saline solution into 10 to 12 Swiss mice, 4 to 5 weeks old. All inoculations were made intraspinally using a technic described by Habel and Li(8). Inoculated mice were observed for paralysis and death for 2 weeks. The brains and cords of several paralyzed mice were studied histopathologically and poliomyelitis type of lesions was found.

Experimental. During the course of our studies with attenuated poliomyelitis viruses, it was realized that in order to maintain the attenuated character of the variant, it would probably be necessary to maintain the virus under standard culture conditions. However, the striking effect of the medium on the virulence of the LH-a virus was discovered by accident. The mouse avirulent LH-a virus was brought from the virus laboratory in Montgomery to the Nat. Inst. of Health, Washington, D.C. in 1955. The virus was then serially passed in tissue culture and inoculated intraspinally into mice. Unexpectedly, some of the mice became paralyzed. At first it was thought that this effect was due to the difference in the strains of the mice used but the

‡ Parke, Davis and Co., Detroit, Mich.

§ Merck, Sharpe & Dohme, Philadelphia, Pa.

CHART 1. Effect of Maintenance Medium of Monkey Kidney Tissue Culture on Mouse Virulence of LH–a Virus.



Key:

T = Monkey testicular tissue culture passage.
K = " kidney " " "
M = Mice inoculated intraspinally.

* No. of $TCID_{50}$ (log 10) in .02 ml fluid inoculated into a mouse.

† No. of mice paralyzed over No. inoculated.

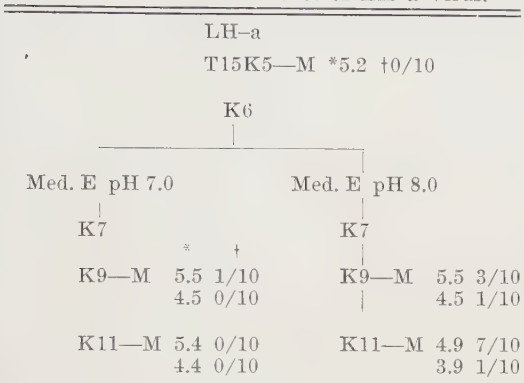
assumption turned out to be incorrect. The other possible explanation was that Medium E was used for tissue culture in Montgomery while Medium 199 was used in Washington. In order to test the effect of the medium on the virulence of the virus, Exp. I was set up which led to a number of experiments, a few examples of which are described below:

Exp. I. Comparison of Medium E and Medium 199. The LH-a virus, T15K4 passage was inoculated into 2 sets of monkey kidney tissue culture tubes. In one set Medium E was used while in the other, 199 medium with 2% calf serum was used. Both media were adjusted to the same pH, 7.4-7.5. Comparable tissue culture fluids of both sets were titrated in mice and in tissue culture. As shown in Chart I the virus from the Medium E set was avirulent for mice, even $10^{5.2}$ $TCID_{50}$ virus (K12) did not paralyze the inoculated mice. Virus from Medium 199, however, was virulent for mice; even $10^{3.2}$ $TCID_{50}$ virus (K15) paralyzed some of the inoculated mice. This mouse virulent variant was designated as LH-v and was used as a testing virus for further experiments. The passages were carried further than that indicated in Chart I and were also repeated many times and similar results were obtained, except that there was slight variation in the effect of 199 media from different sources. It was also shown by further experiments that the presence or absence of calf serum in the medium made no significant difference.

Exp. II. Effect of the pH of medium. Dulbecco, Vogt and Sabin(9) discovered a distinct relationship between neurotropism for the monkey and plating efficiency of poliovirus. We could confirm the effect of the pH of the medium on virulence of the virus but the effect was not in the same direction as shown by these authors, possibly because we were testing virulence in mice rather than in monkeys. One of the experiments along this line was carried out as follows: The virus LH-a, T15K5 passage was inoculated into 2 sets of monkey kidney tissue culture tubes. Medium E was used for both sets but in one set the pH was adjusted to 7.0, in the other to 8.0. Both sets were transferred for 5 passages and the tissue culture fluid from the third (K9) and the fifth (K11) passage were titrated in tissue culture and in mice. The virus from the set with a high pH (8.0) was virulent for mice while the virus from the other set was avirulent (Chart 2). Obviously, in order to test the effect of a given medium in comparison with another, the pH of both media must be kept constant.

Exp. III. Comparison of Eagle's, 199 and Lactalbumin Media. In this experiment the LH-a virus, T15K5, was inoculated into 3 sets of monkey kidney tissue cultures fed with lactalbumin hydrolysate medium, 199 medium, and Eagle's basal medium respectively. The purpose of this experiment was to find out whether there were any media other than Medium 199 which would restore the virulence of the virus. As shown in Chart 3, in the Eagle's basal medium, the virus became highly virulent after 4 passages. In the 199 medium it took 6 passages for the virus to become virulent. In the lactalbumin hydrolysate medium the virus remained avirulent after 6 passages. This experiment demon-

CHART 2. Effect of pH of Medium of Tissue Culture on Mouse Virulence of LH–a Virus.



Key:

T = Monkey testicular tissue culture passage.
K = " kidney " " " .
M = Mice inoculated intraspinally.

* No. of $TCID_{50}$ (log 10) in .02 ml fluid inoculated into a mouse.

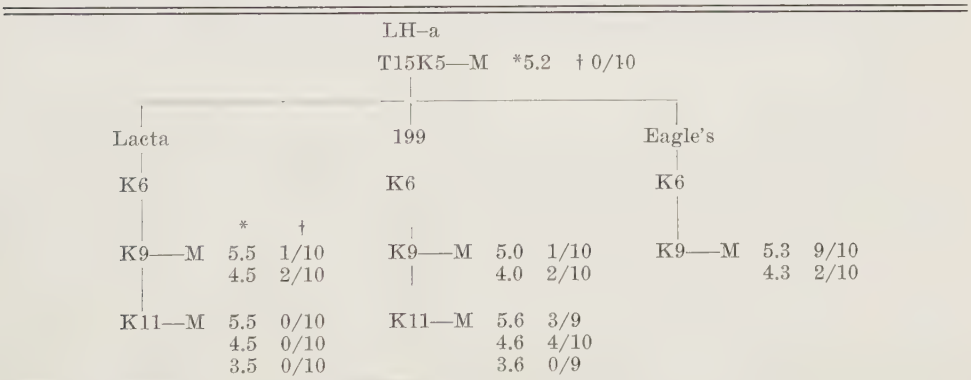
† No. of mice paralyzed over No. inoculated.

strated that both the Eagle basal medium and 199 medium belonged to the virulence enhancing group but the former was more effective. The lactalbumin medium behaved like Medium E.

Exp. IV. Reversibility of LH-v Virus. In the foregoing experiments, it was demonstrated that the virulence of the LH-a virus could be restored by passages in monkey kidney tissue culture fed with Medium 199 or Eagle's basal medium, yielding the mouse virulent variant LH-v. An experiment was conducted to ascertain whether the virulence of the LH-v virus could again be reduced or nullified by changing the medium. The LH-v virus, taken from T15K14 passage in 199 medium (Chart 1) was inoculated into 3 sets of monkey kidney tissue culture tubes overlaid with Medium E, Medium 199 and lactalbumin medium respectively, (Chart 4). Five serial passages were carried out and comparable fluids from each group were titrated in tissue culture and in mice. As shown in Chart 4, the virus became avirulent after 5 passages in Medium E and lactalbumin medium but remained virulent in Medium 199.

Exp. V. Comparison of different cells in the same medium. It was shown previously(1) that the change in virulence was related to the kind of host cells in which the virus was propagated. This relationship was confirmed in the present study and an illustrative example is given below: The LH-v virus from K15 passage (Chart 1) was inoculated into 3 sets of tissue culture tubes. In Set 1, HeLa cells were used, while in Set 2 monkey kidney cells were used, but in both sets the cells were overlaid with the same 199 medium with 2% calf serum. In Set 3, the same monkey kidney cells were used but they were overlaid with Medium E. All the media were adjusted to pH 7.5. Five serial passages in the respective

CHART 3. Comparison of Effect of Eagle's Basal Medium, 199 Medium and Lactalbumin Medium of Tissue Culture on Mouse Virulence of LH–a Virus.



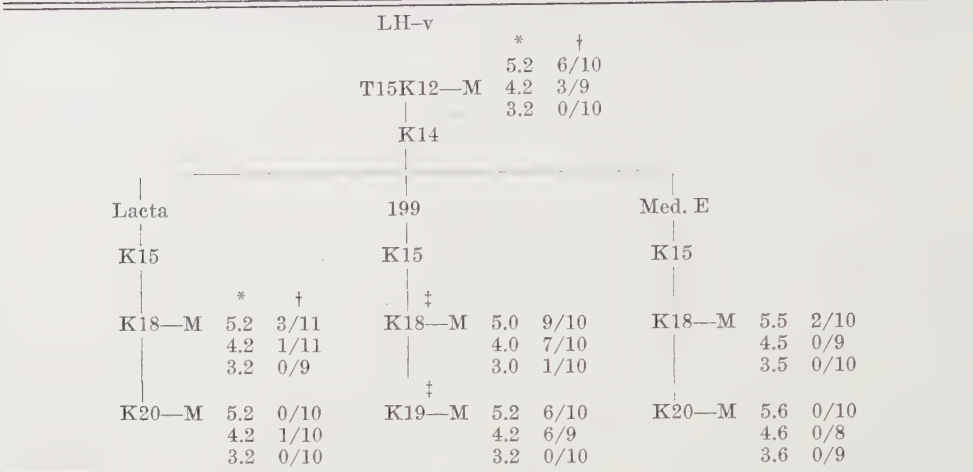
Key:

T = Monkey testicular tissue culture passage. K = Monkey kidney tissue culture passage. M = Mice inoculated intraspinally. Lacta = Lactalbumin medium. 199 = 199 medium. Eagle's = Eagle's basal medium.

* No. of $TCID_{50}$ (log 10) in .02 ml fluid inoculated into a mouse.

† No. of mice paralyzed over No. inoculated.

CHART 4. Reversibility of Mouse Virulence of LH–v Virus Passed in Tissue Culture.



Key:

T = Monkey testicular tissue culture passage. K = Monkey kidney tissue culture passage. M = Mice inoculated intraspinally. Lacta = Lactalbumin medium. 199 = 199 medium.

* No. of $TCID_{50}$ (log 10) in .02 ml fluid inoculated into a mouse.

† No. of mice paralyzed over No. inoculated.

‡ K19 was not as virulent as K10 as a different preparation of 199 medium from a different source was used for K19.

media were carried out, and the harvests from the last 2 passages of each set were titrated in tissue culture and in mice.

In the same host cell (monkey kidney) the virus lost its virulence in Medium E but retained its virulence in Medium 199 (Chart 5). This was the usual finding, as shown in Exp. 4. The striking result was that the virus lost its virulence completely in the HeLa cell culture but retained its virulence in monkey kidney cell culture although the cells in both sets were overlaid with the same medium at the same pH.

Discussion. In studying the effect of various factors on the mouse virulence of the type III poliovirus, it was advantageous to have a strain of readily variable virus so that any slight effect of the testing material on the virulence could be demonstrated. It was also necessary to have two variants; one mouse virulent for testing virulence reducing factors and the other mouse avirulent for testing virulence enhancing factors. The availability of both of these variants (LH-a and LH-v) made this study possible. However, neither variant had been purified, *i.e.,* both represented mixed populations of virulent and avirulent particles, but in the LH-a variant there were more avirulent particles than virulent particles and the reverse was true for the LH-v variant. Further work indicated that purified variants isolated by terminal dilution and the plaque method were less readily variable so they were not good testing tools. On the other hand they could still be made virulent or avirulent by the same measures used in the present study except that it took more serial passages to accomplish the task (10).

In this connection it must be mentioned that continuous passages of the LH-a variant in the virulence reducing system seemed to stabilize the avirulent character of this variant for it took many more passages in a virulence enhancing medium to restore its virulence. Continuous passages of the LH-v variant in the virulence enhancing medium would also make it more virulent and less readily reversible, (*e.g.,* in Medium 199, K15 was more virulent than K12, as shown in Chart 1).

Summary. A method of testing the effect of various factors on the mouse virulence of the type III poliovirus was described. Variants of the virus which could be made virulent or

CHART 5. Comparison of Mouse Virulence of LH–v Virus Passed in Different Host Cells Maintained in Medium 199.

			LH–v					
			T15K12					
			K13					
					*	†		
			K15—M		5.2	7/10		
					4.2	4/10		
					3.2	3/10		
199			199				Med. E	
H1			K16				K16	
	*	†						
H5—M	5.5	0/7	K19—M	5.5	8/10		K19—M	5.5	0/10
	4.5	0/10		4.5	4/10			4.5	1/10
	3.5	0/9		3.5	0/10			3.5	0/10
H6—M	5.4	0/10	K20—M	5.0	8/10		K20—M	5.0	2/10
	4.4	0/5		4.0	3/11			4.0	0/11
	3.4	0/10		3.0	0/11			3.0	0/10

Key:
T = Monkey testicular tissue culture passage. K = Monkey kidney tissue culture passage. H = HeLa cell tissue culture passage. M = Mice inoculated intraspinally. 199 = 199 medium.
* No. of $TCID_{50}$ (log 10) in .02 ml fluid inoculated into a mouse.
† No. of mice paralyzed over No. inoculated.

avirulent were isolated. The virus was designated as LH-v in its virulent phase and LH-a in its avirulent phase. Using these variants as testing tools, 3 factors affecting the viral virulence were found; namely, the host cell, the maintenance medium, and the pH of the medium. These factors were classified into 2 categories, namely the virulence reducing group and the virulence enhancing group. When tested with monkey kidney cell cultures in the pH range 7.0-7.5, Eagle's basal medium and 199 medium belonged to the virulence enhancing group while Medium E (bovine plasma hydrolysate) and lactalbumin hydrolysate medium belonged to the virulence reducing group. When tested in Medium 199 at pH 7.0-7.5 monkey kidney cells enhanced the viral virulence while HeLa cells behaved in the opposite way.

The author wishes to express his thanks to Dr. Ruth Kirschstein of this laboratory who kindly made the histopathological studies and also to Mr. E. C. Martino whose energetic efforts carried the load of technical procedures.

1. Li, C. P., and Jahnes, W. G., *Virology,* 1956, v2, 828.

2. Dulbecco, R., and Vogt, M., *J. Exp. Med.,* 1954, v99, 167.

3. Youngner, J. S., PROC. SOC. EXP. BIOL. AND MED., 1954, v85, 203.

4. Melnick, J. L., and Riordan, J. T., *ibid.,* 1952, v81, 208.

5. Eagle, H., Oyama, V. I., and Levy, M., *Science,* 1956, v123, 845.

6. Eagle, H., Oyama, V. I., Levy, M., and Freeman, A., *J. Biol. Chem.,* 1957, v226, 191.

7. Li, C. P., and Schaeffer, M., *Science,* 1953, v118, 107.

8. Habel, Karl, and Li, C. P., PROC. SOC. EXP. BIOL. AND MED., 1951, v76, 357.

9. Dulbecco, R., Vogt, M., and Sabin, A. B., *J. Am. Med. Assn.,* 1956, v162, 1589.

10. Li, C. P., in preparation.

Received July 26, 1957. P.S.E.B.M., 1957, v96.

Serum Co^{60} Vitamin B_{12} Binding Capacity in Some Hematologic Disorders.*† (23477)

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In normal human subjects, vit. B_{12} is bound to serum protein, probably alpha globulin(1). The serum of these individuals has an additional capacity to bind vit. B_{12} which can be estimated *in vitro* using the radioactive vitamin(2). By microbiological assay, both serum content and binding capacity have been found elevated in chronic myelocytic leukemia, and some cases of polycythemia vera and acute leukemia(3,4). In the present study, the unsaturated binding capacity of serum has been measured in some hematological disorders using Co^{60} vit. B_{12} by a method recently reported from this laboratory (2).

Method. Co^{60} vit. B_{12} having a specific activity of 968 μc/mg was used. To 1 ml aliquots of serum in a Visking bag were added 1, 2.5, 5, 10, 25, 50, or 100 mμg of the radioactive vitamin. After incubating 2 hours at room temperature, the mixture was subjected to exhaustive dialysis in cold, running tap water for 48 hours. "Bound" vit. B_{12} was then measured in terms of residual radioactivity.

Results. Normals. A range of unsaturated binding capacity was derived from studies on 6 normal adults. This was similar to, but generally lower than the binding capacity in normal subjects previously reported(2). In the present inquiry, a single fresh batch of Co^{60} vit. B_{12} was used, while originally the material used was several years old.

Chronic myelocytic leukemia. Sera from 14 patients were examined. Ten showed unequivocal elevation of vit. B_{12} binding capacity, 2 were borderline, and 2 fell into the normal range (Table I). Of the ten, 3 showed marked and 7 moderate elevation (Fig. 1a, b).

Using the binding capacity of 1 ml of serum for 25 mμg of vit. B_{12} as a reference point, the correlation was fair with total leukocyte count, and less good with the absolute myelocyte-myeloblast count (Fig. 1c, d).

Two patients were studied before and after chemotherapy. In patient K, when hematological and clinical remission ensued, Co^{60} vit. B_{12} binding fell to the normal range. When partial remission occurred in patient S, the binding capacity was insignificantly reduced (Fig. 2a). Two patients, C and F, who showed normal binding capacity were in clinical remission, although the former still had a leukocyte count elevated to 41,000/cmm.

Patient J was studied terminally at which time he showed morphological evidence of acute disease. His serum had the highest vit. B_{12} binding capacity in this survey.

Chronic lymphocytic leukemia. Previous reports indicated no significant increase in serum content of vit. B_{12} in this disease(3,4). Of 6 patients studied, serum of one showed moderate, and 2 slight increase in vit. B_{12} binding capacity. The other 3 were in the normal range (Fig. 2b). The binding capacity when abnormal was less elevated than in myelocytic leukemia. Correlation between binding capacity of 1 ml of serum at the 10 mμg level and total leukocyte count was poor.

Acute leukemia. Eight adults in relapse were studied. In 7 the cell type was identified as myeloblastic. Two sera showed an increased vit. B_{12} binding capacity (Fig. 2c). In patient McC the course was subacute, of one year's duration. One person who had acute stem cell leukemia showed a normal serum binding capacity. *Polycythemia vera.* Two patients were studied. One showed mild elevation of serum binding capacity for vit. B_{12} while the other was within the normal

* Co^{60} Vit. B_{12} was supplied by Merck and Co., Rahway, N. J.

† A preliminary abstract was published in *Clin. Research Proc.*, 1957, v5, 143.

Partly supported by U. S. Atomic Energy Commission.

Presented at annual meeting of Society for Nuclear Medicine, Oklahoma City, June 21, 1957.

TABLE I. Co^{60}Vit. B_{12} Binding Capacity of Serum in Chronic Myelocytic Leukemia.

Patient	$m\mu g$ $Co^{60}B_{12}$ bound at 25 $m\mu g$/ml	Current therapy	Total leukocytes per mm^3	Total myelocytes-myeloblasts
Normal avg + 2 S.D.	3.51			
P	6.95	Prednisone	18,900	764
K (pre-therapy)	12.40	6 M-P	117,000	24,570
K (post-therapy)	3.45	6 M-P	5,050	101
Me	3.78	Myleran	73,000	14,600
Mu	4.78	"	22,400	1,568
C	3.10	"	41,000	1,640
M	5.15	"	39,000	3,540
Pa	3.80	None	21,000	6,930
Wi	4.40	"	376,000	92,120
F	2.68	"	11,650	0
Ma	5.60	Myleran	87,000	31,500
S (pre-therapy)	11.83	"	95,000	17,100
S (post-therapy)	10.08	"	47,000	5,640
R	4.23	None	16,500	165
Po	7.40	6 M-P	30,000	3,600
J	20.25	None	73,000	38,690

range. The patient with elevated binding capacity had a persistent leukocytosis of 15-25,000/cmm, while the other patient's leukocyte count remained about 10,000/cmm. *Pernicious anemia.* Serum binding capacity for vit. B_{12} of 3 patients in relapse was normal.

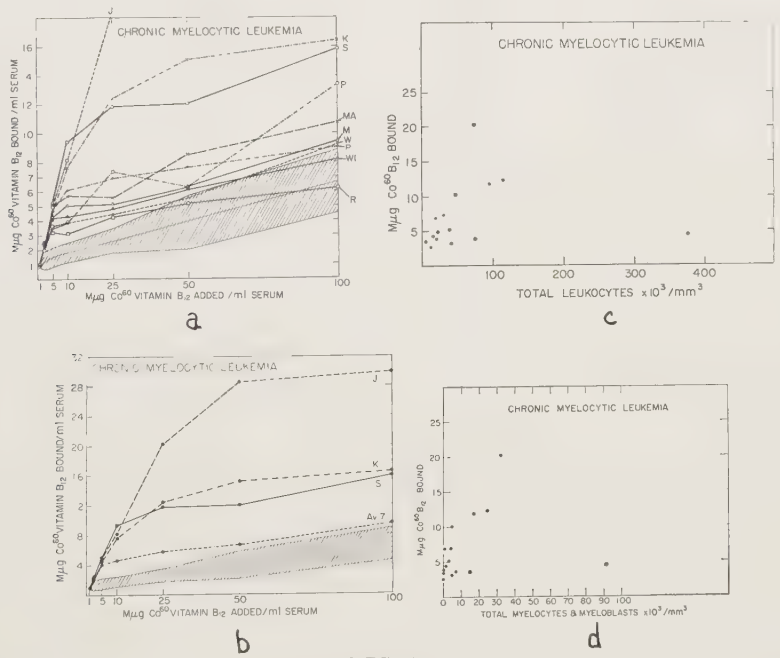

FIG. 1.

(a) Binding capacity for Co^{60} vit. B_{12} of sera from 10 patients with chronic myelocytic leukemia. Shaded area represents normal avg binding capacity $\pm$ 2 S.D.

(b) Serum binding capacity for Co^{60} vit. B_{12} in 3 cases of chronic myelocytic leukemia showing the greatest elevation. Avg of binding capacity in 7 other patients is shown.

(c) Correlation of serum binding capacity for Co^{60} vit. B_{12} in chronic myelocytic leukemia with total leukocyte count.

(d) Correlation of serum binding capacity for Co^{60} vit. B_{12} in chronic myelocytic leukemia with absolute myeloblast-myelocyte count.

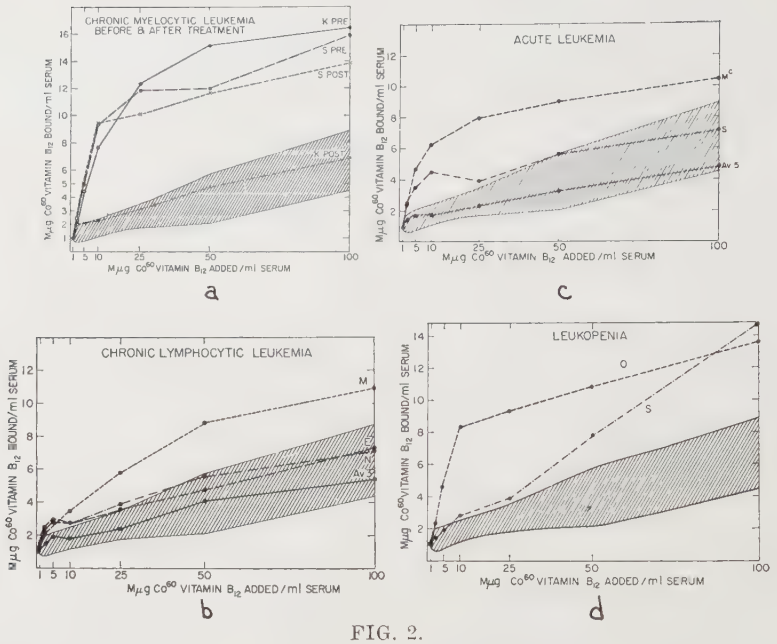

FIG. 2.

(a) Serum binding capacity for Co^{60} vit. B_{12} in chronic myelocytic leukemia before and after therapy.

(b) Serum binding capacity for Co^{60} vit. B_{12} in 3 cases of chronic lymphocytic leukemia showing an elevation. Avg of 3 cases falling within normal range is shown.

(c) Serum binding capacity for Co^{60} vit. B_{12} in 2 cases of acute leukemia showing an elevation and 5 cases (avg) falling within normal range.

(d) Serum binding capacity for Co^{60} vit. B_{12} in 2 cases of idiopathic neutropenia.

Leukopenia. Two adults with chronic, idiopathic neutropenia were studied. The bone marrow of each was normal. Each exhibited abnormally elevated serum binding capacity for vit. B_{12} (Fig. 2d). It will be noted that the pattern of binding of patient O resembles that seen in chronic myelocytic leukemia. In patient S, the abnormal binding is manifest only when the larger amounts of vit. B_{12} are used.

Discussion. Several observers, using microbiological estimation, have noted an increased binding capacity for vit. B_{12} in sera of patients with chronic and acute myelocytic leukemia(3,5). Others, employing radioactive vitamin, have noted a decreased rate of its plasma clearance in chronic myelocytic leukemia(6,7). This may be considered an equivalent *in vivo* phenomenon, because with increased binding capacity a larger part of the administered vitamin is bound to serum protein and consequently lost from plasma more slowly than in normals. In general, the *in vitro* or *in vivo* abnormality showed a mildly positive or no relationship with total leukocyte count and severity of disease. These findings are in harmony with the results of the present study. We have also confirmed (though in one case only) the increased binding capacity which may be present in polycythemia vera.

Inspection of the graph (Fig. 1 a, b) of vit. B_{12} binding in patients with chronic myelocytic leukemia shows a rapidly ascending essentially straight segment followed by a portion which parallels the normal. This suggests a specific binding mechanism which becomes saturated, following which non-specific binding—as in normals—becomes evident. If the straight portions of the ascending and horizontal limbs of the graphs were extended to an intersecting point, this point might be a measure of total binding capacity. We have arbitrarily used the amount of vitamin bound when 10 or 25 mμg were added per ml serum for correlation with leukocyte count.

Because previous investigation had shown increased serum binding capacity for vit. B_{12}

only in those hematological disorders characterized by increased granulopoiesis, Mollin (3) proposed that disintegrating granulocytes might be the source of an increased binding substance in the serum. He was able to demonstrate this *in vitro* by mechanically injuring granulocytes. In the present report, we have shown that the serum of patients with chronic lymphocytic leukemia and idiopathic granulocytopenia may have an increased binding capacity for vit. B_{12}. This suggests an alternative source for binding substance. However, if the rate of granulocyte production were masked by an excessive rate of destruction, these cells might represent the source of binding substance in all instances. Leukocyte agglutinins, which have been reported to be only infrequently present in chronic lymphocytic leukemia and idiopathic neutropenia (8), were not sought in these cases.

Other investigators have noted serum content of vit. B_{12} to be elevated always in chronic myelocytic leukemia, even during remission (3,4,7). Our method does not permit accurate measurement of less than 1 mμg of vitamin. With this reservation, 2 of our patients with this disease showed normal, 2 borderline elevation of vit. B_{12} binding capacity. All 4 were under treatment and in various degrees of clinical and hematological remission. A fifth patient's serum fell to normal in binding capacity following treatment. Patient W, who had received no specific therapy and who had the highest leukocyte count in this study, showed only a moderate elevation of binding capacity. However, she had been treated with parenteral vit. B_{12} for several months before she came to our attention. This may have saturated her binding capacity, as Mollin has shown (3), thus accounting for the relatively small degree of unsaturation demonstrated.

The findings in acute myelocytic leukemia are in accord with previous demonstrations that serum content of and binding capacity for vit. B_{12} in this disease may be elevated, though usually to a lower level than in the chronic form of the disease.

Summary and conclusions. 1. A survey of *in vitro* Co^{60} vit. B_{12} binding capacity of serum from patients with various hematological diseases is presented. Results are compared with previous studies employing microbiological assay and with measurements of serum content as well as binding capacity for the vitamin. 2. These data confirm earlier findings of elevated binding capacity in acute and chronic myelocytic leukemia and in polycythemia vera. 3. An increased binding capacity in some cases of chronic lymphocytic leukemia and idiopathic neutropenia is noted for the first time.

1. Pitney, W. R., Beard, M. F., and Van Loon, E. J., *J. Biol. Chem.,* 1954, v207, 143.

2. Bertcher, R. W., and Meyer, L. M., PROC. SOC. EXP. BIOL. AND MED., 1957, v94, 169.

3. Mollin, D. L., and Ross, G. I. M., *Br. J. Hem.,* 1955, v1, 155.

4. Beard, M. F., Pitney, W. R., and Sanneman, E. H., *Blood,* 1954, v9, 789.

5. Beard, M. F., Pitney, W. R., Sanneman, E. H., Sakol, M. J., and Moorhead, H. H., *Ann. Int. Med.,* 1954, v41, 323.

6. Miller, A., Corbus, H. F., and Sullivan, J. F., *J.C.I.,* 1957, v36, 18.

7. Mollin, D. L., Pitney, W. R., Baker, S. J., and Brodley, J. E., *Blood,* 1956, v11, 31.

8. Payne, R., *Arch. Int. Med.,* 1957, v99, 587.

Received July 29, 1957. P.S.E.B.M., 1957, v96.

Antimetabolites of Mevalonic Acid Utilization in *Lactobacillus acidophilus* 4963.* (23478)

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Mevalonic acid refers to a new compound, beta - methyl - beta, delta - dihydroxy valeric acid (MVA) discovered by Skeggs *et al.*(1) as a growth factor for certain lactobacilli in a medium devoid of acetate, isolated by Wright *et al.*(2) from a large quantity of distillers' solubles, and characterized as described above by Wolf *et al.*(3). The compound has been shown by Tavormina *et al.*(4) to be converted readily to cholesterol by an enzyme system of rat liver. Subsequent studies from several laboratories have confirmed the high order of activity of MVA as an intermediate in cholesterol or squalene biosynthesis and have supplied information on the mechanism of the incorporation(5,6,7,8,9). During the course of some current studies on MVA metabolism various compounds that have been implicated with a relationship to cholesterol biosynthesis in mammalian tissue have been examined for their effect on mevalonic acid utilization by *Lactobacillus acidophilus* 4963, an organism that may be grown on a synthetic medium containing relatively small amounts of MVA or relatively large amounts of acetate. The microbiological results may be of interest for comparative purposes.

Methods and materials. Microbiological determinations were carried out with *Lactobacillus acidophilus* ATCC 4963 essentially as described by Skeggs *et al.*(1). MVA was used in the form of the DL N, N^1-dibenzylethylenediammonium salt.† The extent of bacterial growth was determined with a Klett-Summerson photoelectric colorimeter and the results given are in terms of scale units corrected for the reading of the blanks (no added MVA). Hydroxy methyl glutaric acid (HMG) and dimethyl acrylic acid (DMA, methyl crotonic acid, senecioic acid) were commercial products. Farnesinic acid (FA) #1‡ was synthesized by the Reformatsky re-

TABLE I. Influence of Farnesinic Acid (FA) on Response of *Lactobacillus acidophilus* 4963 to Mevalonic Acid (MVA).

Exp. No.	MVA, γ/10 ml	FA, γ/10 ml	Turbidity
1	0		0
	.1		29
	.2		44
	.3		53
	.4		56
	.6		65
	1.0		75
	.1	100, #1	8
	"	200	0
	"	300	0
	"	500	0
	"	1000	0
	1	100	73
	1	200	65
	1	300	58
	1	500	42
	1	1000	0
	10	100	82
	"	200	80
	"	300	71
	"	500	51
	"	1000	7
2	0		0
	.1		19
	.2		32
	.3		40
	.4		51
	.6		69
	1.0		88
	.1	10, #1	19
	"	20	21
	"	30	19
	"	50	23
	"	100	13
	"	200	0
	"	300	0
	"	500	0
	.1	10, #2	20
	"	20	18
	"	30	22
	"	50	21
	"	100	22
	"	200	13
	"	300	4
	"	500	0

* Supported in part by research grant from National Science Fn.

† Kindly supplied by Dr. Karl Folkers, Merck Sharp and Dohme Laboratories, Rahway, N. J.

‡ Kindly supplied by Dr. James M. Sprague, Merck Sharp and Dohme Laboratories, West Point, Pa.

TABLE I (continued).

Exp. No.	MVA, γ/10 ml	FA, γ/10 ml	Turbidity
3	0		0
	.1		28
	.2		36
	.3		40
	.4		50
	.6		64
	1.0		79
	.1	200, #1	6
	"	400	0
	"	600	0
	.1	200, #2	19
	"	400	4
	"	600	0
	.1	200, #3	18
	"	400	3
	"	600	0

action from geranyl acetone, samples #2§ and #3‖ were prepared from nerolidol through farnesal. α-Phenyl butyric acid (PBA) and α-p-biphenylyl butyric acid (BBA) were synthesized by accepted methods.‡

Results. As indicated by the data of Tables I and II farnesinic acid (FA), α-p-biphenylyl butyric acid (BBA), β-hydroxy-β-methyl glutaric acid (HMG), α-phenyl butyric acid (PBA), and possibly dimethyl acrylic acid (DMA) are all antimeabolites of MVA in decreasing order of activity as listed in the growth of *Lactobacillus acidophilus* 4963. There is some suggestion that FA sample #1 prepared by the Reformatsky reaction and likely to contain a monocyclic byproduct impurity is more active than the other 2 samples. Dituri *et al.*(10) have presented evidence that farnesinic acid is an intermediate between acetate and squalene. Sandermann and Stockmann, on the other hand, have reported(11) that C^{14}-labeled FA prepared by the Reformatsky reaction from geranyl acetone is not converted to C^{14}-cholesterol by rat liver, but sufficient experimental data are not given for a critical evaluation. Evidence that FA is not an intermediate of squalene synthesis in yeast is provided by the results of Amdur *et al.*(6) obtained with doubly-labeled (carbon(C^{14}) and hydrogen(tritium, T)) MVA. This group found that 2-C^{14}-5-di-T-MVA is incorporated into squalene by a particle-free

§ Kindly supplied by Dr. Gilbert Stork, Columbia University, N. Y. City.

‖ Kindly supplied by Drs. Frank Dituri and Samuel Gurin, University of Pennsylvania, Philadelphia.

TABLE II. Influence of Various Compounds on Response of *Lactobacillus acidophilus* 4963 to Mevalonic Acid (MVA).

Exp. No.	MVA, γ/10 ml	Analog, γ/10 ml	Turbidity
1	0		0
	.1		52
	.2		55
	.3		59
	.4		63
	.6		70
	1.0		80
	.1	100 HMG	43
	"	200	38
	"	300	30
	"	400	22
	"	1000	9
	"	2000	4
	"	3000	4
	"	4000	6
	.2	100	56
	"	200	53
	"	300	55
	"	400	55
	"	1000	42
	"	2000	25
	"	3000	19
	"	4000	16
2	0		0
	.1		34
	.2		50
	.3		57
	.4		59
	.6		73
	1.0		84
	.1	100, FA #1	3
	"	200	2
	"	300	0
	"	500	0
	.1	100 HMG	20
	"	200	17
	"	300	12
	"	500	3
	.1	100 DMA	21
	"	200	23
	"	300	20
	"	500	13
	.1	100 PBA	24
	"	200	23
	"	300	24
	"	500	15
	.1	100 BBA	16
	"	200	13
	"	300	11
	"	500	9

FA = Farnesinic acid. HMG = Hydroxy methyl glutaric acid. DMA = Dimethyl acrylic acid. PBA = Phenyl butyric acid. BBA = Biphenylyl butyric acid.

system of yeast with no change in the $T:C^{14}$ ratio. If FA were an intermediate a decrease in the $T:C^{14}$ ratio of 33⅓% would be required since in the formation of FA from MVA the primary alcohol of 1 MVA in 3 is oxidized to a carboxyl group with corresponding loss of all T or H. Wright and Cleland have reported(9) that the conversion of C^{14}-MVA to cholesterol by rat liver homogenates is markedly reduced by the presence of FA. Such an effect would be expected of a compound that is either a precursor or an antimetabolite of MVA. The present microbiological results favor the interpretation that FA is an antimetabolite of MVA utilization. It must be admitted, however, that the fate of MVA in the lactobacillus is unknown and it is conceivable that FA is a metabolite of MVA in the liver system and an antimetabolite in the microbiological system.

PBA and BBA have found some utility as hypocholesterolemic agents, particularly in Europe. Tavormina and Gibbs have studied (12) these compounds as antimetabolites of acetate and mevalonate in the rat liver homogenate system that synthesizes cholesterol and have reported that BBA suppresses acetate and mevalonate incorporation 99 and 93% respectively whereas PBA has no significant effect on acetate or mevalonate incorporation. It is of interest, therefore, that the present microbiological procedure evaluates these 2 compounds in the same order as antimetabolites of mevalonic acid utilization. It is suggested that this microbiological system may have utility in the search for other hypocholesterolemic compounds.

The finding that HMG is an antimetabolite of MVA was not expected. The compound is known to occur in liver but it is no longer considered to be a metabolite in cholesterol synthesis(13). The compound was not found to have significant antimetabolite activity in the liver system(9). DMA, a compound that is incorporated into cholesterol by mammalian tissue at such a low rate as to be considered off the main path of biosynthesis(4), has low to questionable antimetabolite activity.

Summary. Farnesinic acid, α-p-biphenylyl butyric acid, β-hydroxy-β-methyl glutaric acid, α-phenyl butyric acid and dimethyl acrylic acid were found to be antimetabolites of mevalonic acid in this order of decreasing activity for the growth of *Lactobacillus acidophilus* 4963. Implications of these findings are discussed.

1. Skeggs, H. R., Wright, L. D., Cresson, E. L., Mac Rae, G. G. E., Hoffman, C. H., Wolf. D. E.. and Folkers, K., *J. Bact.,* 1956, v72, 519.

2. Wright, L. D., Cresson, E. L., Skeggs, H. R., Mac Rae, G. D. E., Hoffman, C. H., Wolf, D. E., and Folkers, K., *J. Am. Chem. Soc.,* 1956, v78, 5273.

3. Wolf, D. E., Hoffman, C. H., Aldrich, P. E., Skeggs, H. R., Wright, L. D., and Folkers, K., *J. Am. Chem. Soc.,* 1956, v78, 4499.

4. Tavormina, P. A., Gibbs, M. H., and Huff, J. W., *ibid.,* 1956, v78, 4498.

5. Tavormina, P. A., and Gibbs, M. H., *ibid.,* 1956, v78, 6210.

6. Amdur, B. H., Rilling, H., and Bloch, K., *ibid.,* 1957, v79, 2646.

7. Dituri, F., and Gurin, S., *ibid.,* 1957, v79, 2650.

8. Cornforth, J. W., Cornforth, R. H., Popjak, G., and Youhotsky-Gore, I., *Biochem. J.,* 1957, v66, 10.

9. Wright, L. D., and Cleland, M., PROC. SOC. EXP. BIOL. AND MED., 1957, v96, 219.

10. Dituri, F., Cobey, F. A., Warms, J. V. B., and Gurin, S., *J. Biol. Chem.,* 1956, v221, 181.

11. Sandermann, W., and Stockmann, H., *Naturwissenschaften,* 1956, v43, 581.

12. Tavormina, P. A., and Gibbs, M. H., *J. Am. Chem. Soc.,* 1957, v79, 758.

13. Gurin, S., presented in symposium of AAAS, Dec. 1956.

Received July 29, 1957. P.S.E.B.M., 1957, v96.

Enzymatic Proteolysis of Rat Sponge Biopsy Collagen Tissue. (23479)

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The appearance of excessive collagen in degenerative disease and aging in man focuses attention on factors influencing collagen formation and degradation. Thus far, no naturally occurring mammalian collagenase has been proven to exist; the means whereby collagen turnover takes place remains obscure. The rate of resorption of collagen from the involuting post-partum uterus is, however, exceptionally rapid(1) and this suggests the presence of a substance which has collagenolytic properties. Alternately, the rapid postpartum degradation might reflect an alteration in the uterus itself so that, under the influence of hormonal change, it acquires a new susceptibility to less specific proteolytic agents.

The present study had as its objective the evaluation of the effects of certain enzymes upon the saline-insoluble fraction of rat sponge-biopsy connective tissue(2) at pH 6.8-7.8. The degree of collagenolysis was compared in biopsy-connective tissue removed from untreated rats and from rats treated with estrogen and relaxin prior to removal of the tissue.

Method. Sponge-biopsy connective tissue was obtained from 5 male, 6 female and 2 castrate adult Sprague-Dawley rats. Polyvinyl alcohol sponges had been implanted in the rats from 3 to 8 months before biopsy. Premarin,® 0.025 mg, and relaxin,† 4 mg, were injected subcutaneously into 7 of the rats for 21 consecutive days prior to the removal of the sponge biopsies. The remaining 6 rats received no therapy and served as controls.

The biopsied sponges were sliced into distilled water and homogenized at medium speed in a VirTis homogenizer. Connective tissue was grossly separated from the sponge material after high speed centrifugation. It was then stirred vigorously with 0.1 N saline for two 10-minute periods to remove saline-soluble collagen and non-collagenous proteins. The insoluble residue served as the substrate for the enzymatic studies. Fresh rat-tail collagen, which served as a control collagen, was prepared by stripping out the tendons from an untreated rat, cutting them into short lengths, and treating them similarly to the sponge-biopsy material. All preparative work was done in a cold room or refrigerated centrifuge at 5°C.

200-300 mg aliquots of freshly prepared biopsy tissue were shaken at 37°C for 12 hours with 1 ml buffered solutions containing Trypsin 2 mg, Chymotrypsin 3 mg, Papain 1 mg, Elastase (pure) 1 mg, Hyaluronidase 3 mg,‡ or Elastase (impure) 3 mg.§ All enzymes were dissolved in 0.1 M phosphate buffer, pH 7.8, except Papain (Versene buffer, pH 6.0, containing 0.005 M of Cysteine) and Trypsin (0.1 M phosphate buffer, pH 6.8). Tissues incubated with each buffer but without enzyme served as controls for each animal. As further control, a sample of each enzyme was heated to 100°C for 10 minutes and incubated with the tissue of one rat.

After incubation, the tissues were centrifuged in the cold at approximately 70,000 x g for 40 minutes. The supernatants were passed through sintered glass filters to remove any remaining particulate collagen. Aliquots of each supernatant were evaporated to dryness and hydrolysed in 1 ml of 6 N HCl at 110°C for 16 hours. The residues were hydrolysed similarly. The collagen concentration of each fraction was determined by the

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† Relaxin was supplied through the courtesy of the Warner-Chilcott Laboratories.

‡ Trypsin, 2 x crystalline, Chymotrypsin, 2 x crystalline, Papain, 2 x crystalline, Hyaluronidase, 100-200 TRU/mg—Nutritional Biochemicals Co.; Elastase, 2 x—Worthington Biochemicals Corp.

§ Elastase, impure, was supplied through the courtesy of the Warner-Chilcott Laboratories.

TABLE I. Enzymatic Digestion of Collagen.

Enzyme	Collagen digested, % of total collagen: Untreated control	Treated	Hydroxyproline, mg/100 mg protein in residue
Buffer, pH 6.8	.37 ± .08	.60 ± .35*	6.54 ± .39
pH 7.8	.28 ± .09	.32 ± .12	6.36 ± .54
Chymotrypsin 2×	2.74 ± .71	2.29 ± .38	8.66 ± .37
Trypsin 2×	2.99 ± .87	3.85 ± .76	7.64 ±2.12
Papain 2×	3.76 ± .74	2.85 ± .84	8.30 ± .30
Hyaluronidase 100-200 TRU	.59 ± .23	.66 ± .30	—
Elastase 2×†	1.11	1.42 .99	—
Trypsin 2× & elastase 2×	3.69	4.18 3.34	—
Elastase (impure)	10.03 ±1.56	9.77 ±2.51	9.95 ± .54

* Mean and stand. dev.
† Only 3 samples assayed.

hydroxyproline technic of Neuman and Logan (3) and the digested collagen in the supernatant expressed as a percentage of total collagen of the sample. The total protein content of each residue was derived from the nitrogen content as determined by the Conway(4) method after Kjeldahl digestion.

Results. Enzyme incubation of the biopsy-connective tissue from the rats pretreated with estrogen and relaxin resulted in collagen digestion which was not statistically different from those obtained with tissues of the untreated animals (Table I). Because the connective tissues of the male, female and castrate rats were equally susceptible to all the enzymes, the results were calculated and compared on the basis of treatment and control.

Tissue incubated with buffer alone or with heat-inactivated enzyme gave virtually no collagen digestion. Hyaluronidase, a non-proteolytic enzyme, similarly released an insignificant amount of collagen and, in effect, constituted a further control. Trypsin, Chymotrypsin and Papain, on the other hand, gave small but consistent digestion of collagen in all samples. These enzymes resulted in from 2.29 to 3.85% digestion in the two groups of rats. There was no statistically significant difference among the results for these 3 enzymes. Pure Elastase gave approximately 1% digestion, and when combined with crystalline Trypsin gave a mean value of 3.74% digestion for 3 tissues which was not statistically different from that obtained with Trypsin alone. Impure Elastase, on the other hand, resulted in from 6.0 to 18.7% digestion (mean 9.9%) for the 2 groups of animals. Incubation of rat-tail tendon with impure Elastase resulted in the release of 8.9% of total collagen, which is an amount comparable to that obtained with the biopsy-tissue.

The concentrations of hydroxyproline per unit of protein in the connective tissue residues after digestion with Chymotrypsin, Papain or impure Elastase were statistically greater ($p<0.05$) than those in the tissues incubated without enzyme. The large spread of data obtained for the Trypsin-digested residues nullified any significant difference from the controls.

Comments and summary. Pretreatment of rats with estrogen and relaxin does not appear to alter the susceptibility of sponge-biopsy connective tissue to digestion by proteolytic enzymes at near physiologic pH. No difference was noted in the proteolysis of collagen obtained from either sex or from castrate rats. Trypsin, Chymotrypsin and Papain all have a slight proteolytic activity on pretreated or untreated tissue, while pure Elastase and Hyaluronidase effect little, if any, liberation of collagen. Impure Elastase dissolves 2 to 6 times the amount of collagen digested by the proteolytic enzymes or a combination of pure Elastase and Trypsin. It is postulated that the impure Elastase, prepared from pancreas, contains a collagenolytic enzyme distinct from the proteolytic enzymes of the pancreas. The removal of a portion of the scleroprotein of connective tissue by enzyme action results in a residue richer in hydroxyproline (per unit protein) than in the enzyme-free controls. This may occur either through the removal of a non-collagenous, saline-insoluble protein liberated by the enzyme, or through the selective removal of a type of collagen which has a lower hydroxyproline concentration than the tissue as a whole.

1. Harkness, M. L. R., Harkness, R. D., and Mora-

lee, B. E., *Quart. J. Exp. Physiol.,* 1956, v41, 254.

2. Boucek, R. J., and Noble, N. L., *A.M.A. Arch. Path.,* 1955, v59, 553.

3. Neuman, R. E., and Logan, M. A., *J. Biol. Chem.,* 1950, v186, 549.

4. Conway, E. J., *Microdiffusion Analysis and Volumetric Error,* Ed. 3, 1950, p124, Crosby Lockwood and Son Ltd., London.

Received July 30, 1957. P.S.E.B.M., 1957, v96.

Ovulation in Persistent-Estrous Rats after Electrical Stimulation of the Brain.*† (23480)

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The fact of neural regulation of ovulation in rabbits and certain other animals classed as "reflex ovulators" has been well known for many years. Direct evidence has been obtained by the induction of ovulation through electrical stimulation of the hypothalamus(1, 2). More recently, ovulation has been reported in rabbits and cats after stimulation of the amygdaloid complex(3,4). Indirect evidence indicates that *spontaneous* ovulation as exemplified by the rat is likewise regulated by the hypothalamus(5). However, until very recently there was no direct evidence for this from stimulation of the brain. Critchlow(6) is the first to demonstrate it, using proestrous rats in which the "spontaneous" mechanism was blocked out with pentobarbital. To be effective, his electrodes had to be near the median eminence. To produce ovulation in rats by stimulation elsewhere in the nervous system, other means are required to block the spontaneous intrinsic stimulus. One such method is to subject the rat to continuous illumination for a few weeks(7,8,9). In time such an animal usually enters a state of persistent estrus. She can ovulate in response to coitus(9) and in this respect is like the rabbit, cat, etc. For comparison with them she would theoretically serve as a more suitable preparation than the pentobarbital-blocked rat. We wish to record the fact that such preparations can be made to ovulate by electrical stimulation of the brain through electrodes implanted at a distance from the hypothalamus.

Materials and methods. Adult female albino rats over 6 months old were exposed to continuous illumination until a persistent vaginal estrus had become established. Of this group, 12 rats formed the eventual series of acceptable experiments. A bipolar electrode was placed in the brain of each animal by use of a Horsley-Clarke stereotaxic instrument and Krieg's coordinates. The electrodes were fashioned from 0.32 mm Nichrome wire, stretched, and insulated with baked enamel. The spacing of the two wires at the tip was <0.05 mm. In 11 rats the electrode was aimed for the amygdaloid complex; in the remaining rat it was inserted into the septal region. It was fixed to the skull by a bridge made of an 18 mm wound clip clamped to the temporal crests and filled-in beneath with zinc dental cement. The operation was performed under ether anesthesia to allow rapid recovery. Soon afterward the rat was placed in a shoulder harness to which wires from the electrode were anchored before leading off to the stimulator. She had free range of movement within the radius of the wires. Stimulation was carried out after recovery from the anesthetic. The source of stimulus was an electronic impulse generator with independently controlled frequency and intensity sec-

* A portion of a thesis submitted by J. P. B. in partial fulfillment of requirements for the degree of Bachelor of Science in Medicine in the School of Medicine of Duke University.

† Supported in part by a grant from the Research Council of Duke University.

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TABLE I. Summary of Brain Stimulations and Results.

Electrode		Result		
Location	Proof	Interval	Evidence	Score
Amygdala	Sec*	24 hr	11 tubal ova	+
"	Gr obs†	"	12 *Idem*	+
"	"	"	9 "	+
"	Sec	"	9 "	+
"	"	"	8 "	+
"	Gr obs	11 day	1 mm corp lut	?
"	Sec	"	*Idem*	?
"	Gr obs	24 hr	No tubal ova	—
"	"	"	*Idem*	—
"	"	"	"	—
"	Sec	5 day	No corp lut	—
Septum pellucidum	"	48 hr	Many new corp lut	+

* Stained sections of formalin-fixed brain.
† Gross observation of formalin-fixed brain (see text).

tions. The wave form which it delivers is a rapidly rising spike from a condenser discharge, with exponential decay during ca. 3 msec. The frequency and intensity varied from individual to individual. The practice was to administer as much as the animal would take, short of convulsions, during periods of 30-60 minutes. The usual frequency was 25-30 c/sec. The voltage ranged as high as 10-15 v, reaching 20 v in one instance. The instrument was not always dependable, however, and more reliance was placed on the overt response of the animal than on the setting of the voltage dial. The results were assessed at autopsy, which was carried out on the first day after stimulation in 8 rats, on the second day in 1 rat, on the fifth day (proestrus) in 1 rat and on the eleventh day of anestrus in 2 rats. In the 8 rats the fallopian tubes were searched for ova and the ovaries were examined for freshly ruptured follicles(5). In the others, if corpora lutea were present, the ovaries were serially sectioned for histological examination. The intact brain of each animal was preserved entire in 10% formalin. Six were imbedded in celloidin, sectioned at 26 μ and stained by the Luxol fast blue-Cresyl fast violet technic of Klüver and Barrera(10). Otherwise, only gross observations were made. Usually a spot of discoloration could be seen on the ventral surface, marking the location of the electrode tip. If doubt existed, progressive thin slices were made with a razor blade until the electrode track was found.

Results. The results of all acceptable experiments are assembled in Table I. In 11 rats the tips of the electrodes rested in the amygdaloid complex. Five of them had tubal ova on the day after stimulation. Two others possibly ovulated, but the information is indirect on account of the delayed autopsy time. Four failed for no evident reason. In one rat the electrode was aimed at the septum pellucidum; sections of brain later demonstrated that the tip lay in the lateral ventricle impinging on the dorsal lateral aspect of the septum and also in contact with the caudate nucleus. Two days after stimulation her ovaries had new corpora lutea about 36 hours old, as judged from their histological appearance.

Proximity of amygdala and lateral hypothalamus makes it possible that in several cases the positive result may have come about through spread of the stimulating current into the hypothalamus. However, the one rat that was stimulated in the septal region and one of the amygdala group (#2) can hardly be explained on that basis. In the former the distance to the hypothalamus would be too great. In the latter there were few motor signs, in spite of the fact that the electrode tips were just lateral and ventral to the internal capsule. Spread would thus appear to be ruled out.

Seven faulty experiments, in which the electrode was not firmly attached to the skull or the electrical connections were poor or the electrode was not deep enough, may be considered as controls in the sense that they show that mere insertion of the electrode does not serve as an adequate stimulus. None of them ovulated.

The results confirm by a somewhat different method the observation by Critchlow(6) that ovulation can be induced in rats by electrical stimulation of the brain—this in a species that normally ovulates spontaneously. Although the present experiments are only preliminary, they show clearly that the persistent-estrous rat in continuous illumination is a suitable experimental animal for explora-

tion of neural elements of the LH-release apparatus higher than the median eminence. The chief limitation would appear to be the fact that in some strains of rats (*e.g.* our "normal" strain derived from Osborne-Mendel stock) the persistent-estrous state often cannot quickly be induced until the animal is 6 months old.

Summary. Persistent estrus was induced in female rats by continuous illumination. Through "permanent" bipolar electrodes, stimulation was administered after recovery from anesthesia to either the amygdala (11 rats) or the septum pellucidum (1 rat). The latter and 5 of the former ovulated within 24 hours.

1. Harris, G. W., *The Neural Control of the Pituitary Gland.* Edward Arnold, Ltd., London, 1955, 298 pp.

2. Markee, J. E., Everett, J. W., and Sawyer, C. H., *Recent Progress in Hormone Research,* 1952, v7, 139.

3. Koikegami, H., Yamada, T., and Usei, K., *Folia psychiatr. Neurol. Japonica,* 1954, v8, 7.

4. Shealy, C. N., and Peele, T. L., *J. Neurophysiol.,* 1957, v20, 125.

5. Everett, J. W., *Ciba Foundation Colloquia Endocrinol.,* 1952, v4, 167.

6. Critchlow, B. V., *Anat. Rec.,* 1957, v127, 253.

7. Hemmingsen, A. M., and Krarup, N. B., *Kgl. Danske Vidensk. Selsk., Biol. Medd.,* 1937, v13, no. 7, 1.

8. Browman, L. G., *J. Exp. Zool.,* 1937, v75, 375.

9. Dempsey, E. W., and Searles, H. F., *Endocrinology,* 1943, v32, 119.

10. Klüver, H., and Barrera, E., *J. Neuropath. and Exp. Neurol.,* 1953, v12, 400.

Received July 30, 1957. P.S.E.B.M., 1957, v96.

"Transfer" of Complement from One Antigen-Antibody Complex to Another.*† (23481)

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It has been shown that some of the I^{131} labelled complement incorporated with antigen-antibody-I^{131} complement precipitates is released when these precipitates are resuspended in fresh sera containing active complement(1). This behavior was attributed to an equilibration between the I^{131} complement in the precipitates and the free complement present in the fresh sera. It was of interest to determine whether the complement components still maintain their original activities after the reaction with an immune complex. There is some evidence in the literature indicating that complement components once fixed may maintain some of the original ability to participate in the hemolysis of sensitized erythrocytes(2,3).

The purpose of the present investigation was to determine whether or not components of complement can "transfer" from one immune complex to an unrelated one and if so, what are the conditions which govern this "transfer." As an index of complement activity, use was made of the precipitation of soluble antigen-antibody complexes by complement(4,5). Antigen-antibody complexes (soluble or insoluble) containing complement were mixed with soluble I^{131} bovine serum albumin (I*BSA)-rabbit anti BSA complexes which were prepared in the region of excess antigen. The subsequent precipitation of the soluble I*BSA-anti BSA complexes was employed as a measure of the "transfer" of complement to these complexes. Although complement appears to be responsible for the precipitation of soluble immune complexes by normal sera, there is a possibility that other substances present in the sera may play a role. It has previously been inferred that

* Publication No. 133 of Department of Pathology, University of Pittsburgh School of Medicine.

† Presented in partial fulfillment of requirements of degree of Doctor of Philosophy by William O. Weigle, University of Pittsburgh, Pa. Supported in part by Atomic Energy Commission Contract.

nitrogenous materials other than complement may fix to immune precipitates(1).

Materials and methods. BSA was obtained from Armour and Co., lot No. 128-175, egg albumin (Ea) was prepared by the method of Kekwick and Cannan(6). Pneumococcal polysaccharide S III was kindly supplied by Dr. M. Heidelberger. I^{131} BSA (I*BSA) was prepared according to the method described by Talmage *et al.*(7). Sufficient counts were obtained in well type gamma counters to insure a maximum counting error of no greater than 5%. Anti BSA and anti Ea were prepared by intravenous injections of rabbits four times per week with neutral suspensions of alum precipitated protein as previously described(8). The globulin fraction of these antisera was precipitated at 40% saturation with ammonium sulfate, and dialyzed against 0.15 M NaCl until free of sulfate. The antibody content of the fractionated preparations of the anti BSA and anti Ea sera was 1.58 and 2.00 mg N per ml, respectively. These preparations were maintained as stock solutions and diluted for use. Rabbit anti S III, A66, was kindly supplied by Dr. M. Heidelberger. A pool of serum obtained from 30 guinea pigs was used as the source of complement. The serum was placed in a polyethylene container and stored in a dry ice chest at −50 to −70°C without preservatives. The serum was adjusted to pH 7.5 before use. The reagents and methods employed for the titration of the components of complement were those previously described(9,10). Sheep erythrocytes collected in Alsever's solution (11) were obtained from Carworth Farms, New City, N. Y. and the rabbit hemolysin was procured from Lederle Labs, lot No. 2409-132 A. The quantitative immunochemical procedures used were those developed by Heidelberger and co-workers(10). The total N in immune precipitates and in the various protein solutions was measured by the Markham modification of the micro Kjeldahl technic(12). Complement N was recorded as the difference between the total N precipitated (antibody + antigen + complement) in the presence of complement and the total N precipitated in the presence of 0.15 M NaCl or inactivated serum (antibody + antigen). Sera were inactivated by heating for one hour at 56°C. Two ml of guinea pig complement were reacted with mixtures of S III polysaccharide + anti S III and Ea + anti Ea formed at equivalence. The μg of total N in the S III-anti S III and Ea-anti Ea precipitates formed in the absence of complement were 110 and 105 respectively. The antigen-antibody-complement mixtures were incubated at 37°C for 30 minutes and then for various times (1 hour-6 days) at 0-3°C. After different periods of incubation the precipitates which formed were centrifuged and washed twice. Subsequent addition of the antibody-antigen-complement complexes to soluble I*BSA-anti BSA complexes which had been formed at 2 times equivalence and contained 14.5 μg of I*BSA N was performed in 2 different ways. One method consisted of resuspending the antigen-antibody-complement precipitates in the I*BSA-anti BSA complexes. The other involved dissolving the antigen - antibody - complement precipitates with excess antigen (either 2.15 mg S III or 14.70 mg Ea) at 37°C for 30 minutes, centrifuging the mixture at 0°C and adding the clarified solution to I*BSA-anti BSA soluble complexes. The soluble I*BSA-antiBSA complexes and the solutions of redissolved precipitates were present in a volume of 1 ml. These experiments were performed using veronal buffer pH 7.4(10) as a diluent both with and without added Ca^{++} and Mg^{++}. The total N precipitated by the transferred complement was also measured.

Results. The results in Table I show that

TABLE I. Precipitation of I*BSA-Anti BSA Soluble Complexes by Antigen-Antibody-Complement Complexes.

Antigen-antibody‡ complement Ppts.	C'N in Ppts., μg	I*BSA N Pptd. by Ag.-Ab.-C' Ppts.	I*BSA N Pptd. by dissociated Ag.-Ab.-C' Ppts.
S III-anti S III* (156.6 μg N)	51.2	1.6 (11)§	4.7 (32)
Ea-Anti Ea† (162.8 μg N)	60.8	2.6 (18)	3.0 (21)

* 2.15 mg S III used to dissociate precipitate.
† 2.35 mg Ea N " " " "
‡ Used after 6 hr reaction time at 0-3°C.
§ The figures in parentheses are % of available I*BSA (14.5 μg N) which precipitated.

TABLE II. Dissociation of Antigen-Antibody-Complement Precipitates by Excess Antigen after Incubation at 0-3°C.

Time of incubation (0-3°C)	Total N Pptd. (μg) in presence of 2.0 ml of G.P. serum	2.0 ml 0.15 M NaCl	C'N Pptd., μg	Amt of antigen used to dissociate Ag.-Ab.-C' Ppts., mg	N in Ppts. (μg) after dissociations (a)	(b)	% dissociation of Ppts. (a)	(b)
S III-anti S III system								
3 hr	145.0	96.0	49.0	2.15	26.0	14.0	82	85
6 hr	153.4	99.2	54.2	"	28.2	18.2	82	82
24 hr	158.6	100.2	58.4	"	35.0	19.4	78	81
6 days	162.8	106.8	56.0	"	42.4	21.8	74	78
Ea-anti Ea system								
3 hr	151.8	108.2	43.6	14.70	64.6	8.2	58	82

(a) Precipitates initially formed in G.P. serum.
(b) " " " " 0.15 M NaCl.

complement from both antigen-antibody-complement precipitates and soluble antigen-antibody-complement complexes can precipitate soluble I*BSA-anti BSA complexes. The precipitation of these soluble complexes may be the result of the "transfer" of either free complement or complement in the form of antigen-antibody-complement complexes. However, it will be shown later that it is probably free complement which is transferred.

Before investigating the effect of the time of incubation of the antigen-antibody-complement complexes on the ability of complement to be transferred to a second antigen-antibody complex, it was necessary to study the resolution behavior of antigen-antibody precipitates with excess antigen. The results of the resolution of specific precipitates consisting of antibody, antigen and complement and of antibody and antigen (no complement) are given in Table II. The effect of the time of incubation of S III-anti S III precipitates (either with or without complement) at 0-3°C on resolution with excess antigen was not very pronounced. As the time of incubation of the precipitates was increased (from 3 hours to 6 days) they became only slightly more difficult to dissolve with excess S III. With the S III-anti S III system, the presence of complement had very little, if any, effect on the resolution of the precipitates by excess S III. However, with the Ea-anti Ea system, there was a pronounced effect of the presence of complement on the resolution of the precipitates by excess antigen. Eighty-two percent of the total precipitate formed in 0.15 M NaCl was dissolved with excess antigen, whereas, only 58% of the precipitate containing 43.6 μg of complement N was dissolved by the same amount of Ea.

The "transfer" of complement (nitrogen) from dissolved S III-anti S III-complement precipitates as evidenced by the precipitation of soluble I*BSA-anti BSA complexes is shown in Table III. This "transfer" of complement (nitrogen) was inhibited significantly by the addition of Ca^{++} and Mg^{++}. Precipitation of soluble I*BSA-anti BSA complexes occurred in the absence of Ca^{++} and Mg^{++}. Similar results were obtained with the dissociated Ea-anti Ea-complement precipitates (Table IV). When soluble I*BSA-anti BSA complexes were incubated with either S III-anti S III or Ea-anti Ea complexes which lacked complement there was no precipitation of I*BSA.

Precipitates containing complement had similar effects on the precipitation of soluble complexes as did the solutions of the dissolved antigen - antibody - complement precipitates (Tables III and IV). As mentioned above, this precipitation was also inhibited by Ca^{++} and Mg^{++}.

The S III-anti S III-complement precipitates obtained after 3 hours incubation at 0-3°C were dissolved with excess S III both in the presence and absence of Ca^{++} and Mg^{++} and were then analysed for component activities of complement. Only C'1 activity was found in the solutions of the dissolved precipitates. However, 3 times as much C'1 activity was found in the solution prepared in the ab-

TABLE III. Precipitation of Soluble I*BSA-Anti BSA Complexes by S III Polysaccharide-Anti S III-Complement Complexes.

	Time of incubation of S III-anti S III-C′ Ppts. (0-3°C)	Dissociated Ppts. Total N Pptd., μg	I*BSA N Pptd.,† μg	C′N Pptd.,* μg	Undissociated Ppts. Increase in N of S III-anti S III-C′ Ppts.,‡ μg	I*BSA N Pptd.,* μg
In absence of Ca^{++} and Mg^{++}	3 hr	21.8	2.7	6.9	13.2	3.3
	6 hr	37.6	4.4	12.7	15.2	3.4
	24 hr	43.4	4.9	16.5	16.2	3.3
	6 days	31.2	2.8	15.8	7.0	2.8
In presence of Ca^{++} (0.00015 M) and Mg^{++} (0.0005 M)	3 hr		.8		.0	.5
	6 hr		.7		.0	.9
	24 hr		1.3		.0	.5
	6 days		.2		.0	.3

* Obtained by subtracting the antibody N and I*BSA N Pptd. from the total N Pptd. An antibody N/antigen N ratio of 4.5 was used to determine antibody N Pptd.

† I*BSA-anti BSA soluble complexes contained 14.5 μg I*BSA N.

‡ Determined by difference in N content of S III-anti S III-C′ Ppts. before and after incubation with I*BSA-anti BSA soluble complexes.

sence of Ca^{++} and Mg^{++} than in the one prepared in the presence of the divalent cations.

Discussion. It has been observed that complement and possibly complement containing complexes can be transferred from one immune complex to an unrelated one. It is of interest that this phenomenon failed to take place in the presence of concentrations of Ca^{++} and Mg^{++} which have been shown to be necessary for the fixation of hemolytic complement(13-15). Mayer and co-workers(16-18) have postulated a sequence of steps involved in complement fixation and lysis of sensitized cells. They found that Ca^{++} was required for the fixation of C′1 and C′4 while Mg^{++} was required for the fixation of C′2. In the present studies it has been shown that the divalent cations Ca^{++} and/or Mg^{++} inhibited the ability of complement to be "transferred" from one antigen-antibody complex to another. From these results it appears that divalent cations are not only required for the fixation of guinea pig complement to antigen-antibody complexes, but may participate in maintaining a firm bond between complement and the complexes. Laporte *et al.*(19) postulated that complement may exist in the serum as a Ca-proteinate complex. When Ca^{++} was removed from serum they found that complement became more susceptible to inactivation at 37°C. It is possible that complement in the form of a Ca-proteinate maintains a stronger bond with antigen-antibody complexes than "cation free" complement. If this were true, it could readily explain the inhibiting effect of divalent cations on complement

TABLE IV. Precipitation of Soluble I*BSA-Anti BSA Complexes by Ea-Anti Ea-Complement Complexes.

	Total N Pptd., μg	I*BSA N Pptd.,† μg	C′N Pptd.,* μg	Increase in N of Ea-anti Ea-C′ Ppts.,‡ μg	I*BSA N Pptd.,* μg
In absence of Ca^{++} and Mg^{++}	9.7	1.4	3.4	13.0	2.9
In presence of Ca^{++} (0.00015 M) and Mg^{++} (0.0005 M)		.4		.0	.7

Ea-anti Ea precipitates were incubated for 3 hr at 0-3°C prior to dissociation and addition to soluble I*BSA-anti BSA complexes.

* Obtained by subtracting the antibody N and I*BSA N Pptd. from the total N Pptd. An antibody N/antigen N ratio of 4.5 was used to determine antibody N Pptd.

† I*BSA-anti BSA soluble complexes contained 14.5 μg I*BSA N.

‡ Determined by difference in N content of Ea-anti Ea-C′ Ppts. before and after incubation with I*BSA-anti BSA soluble complexes.

"transfer." It may well be that the precipitation of soluble I*BSA-anti BSA complexes in the presence of complement containing complexes is the result of the fixation of antigen-antibody-complement complexes by the soluble I*BSA-anti BSA complexes rather than the result of the "transfer" of complement from one complex to another. However, it appears that divalent cations would be more likely to inhibit the release of complement from immune precipitates than the fixation of complement. It was not determined whether both Ca^{++} and Mg^{++} were necessary to inhibit the "transfer" of complement from one complex to another. However, it appears from the work of Mayer and co-workers that Ca^{++} should be the responsible cation.

The complement released from the S III-anti S III-complement precipitates by excess S III was found to be hemolytically active C'1. This release of hemolytically active complement (C'1) was not surprising in view of the findings of Hill and Osler(3), who demonstrated a return of hemolytic activity after treatment of precipitates containing complement with 10% NaCl. These results are also in accord with the previously reported data(5) that the fixation of hemolytic complement by soluble complexes decreased with an increase in the concentrations of antigen added. Thus, the dissociation, by excess antigen, of precipitates containing complement should be expected to release complement. It is of interest that some of the complement (C'1) released in this manner still retained its hemolytic activity.

From the results presented in Table II, it appeared that complement which had fixed to Ea-anti Ea precipitates retarded the dissociation of the precipitates. This effect of complement on dissociation with excess antigen is similar to the "order of addition" effect reported by Neff and Becker(20). They found that when antibody was added to antigen and incubated for 2 days, less N was precipitated in the presence of complement than when the antigen was added to antibody. However, if the mixtures were incubated for a longer period of time (7 days), or the reaction performed in the presence of decomplemented sera this effect was diminished. The explanation advanced was that when antibody is added to antigen, the complexes are initially formed in the region of antigen excess and complete precipitation of antibody is not obtained until complete equilibrium is established. Neff and Becker showed that complement increased the time necessary for equilibrium to be reached. Likewise in the present experiments, complement inhibited the establishment of equilibrium when excess antigen was aded to Ea-anti Ea-complement precipitates. There are a number of reports in the literature demonstrating that complement retards the flocculation of antigen and antibody (21-23). This may also be attributed to the ability of complement to increase the time for equilibrium to be established.

Boyd(24) found that Ea-anti Ea precipitates incubated at 0°C for 10 months were dissolved with excess antigen to the same extent as "fresh" precipitates. He believed that this demonstrated that the strength of the bonds involved in antigen-antibody precipitates did not increase with time. The data in Table II demonstrate that S III-anti S III precipitates became only slightly more difficult to dissociate with excess S III as the time of incubation (1 hour-6 days at 0-3°C) was increased.

Summary. The "transfer" to soluble I*BSA-anti BSA complexes of guinea pig complement which had previously been fixed to S III-anti S III and Ea-anti Ea precipitates has been demonstrated. This "transfer" of complement was achieved if the complement containing precipitates were added directly to the complexes or first dissociated with excess antigen. It was found that Ca^{++} and Mg^{++} inhibited this "transfer" of complement. The only detectable component of complement released from antibody-antigen-complement precipitates by excess antigen was hemolytically active C'1. Complement which had been fixed to Ea-anti Ea precipitates retarded the establishment of the equilibrium between antibody and antigen when excess antigen was added to these precipitates. It was also shown that the time during which precipitates (antibody + antigen or antibody + antigen + complement) were incubated had only a minor effect on the rate of dissociation of the

precipitates with excess antigen.

1. Weigle, W. O., and Maurer, P. H., *J. Immunol.*, 1957, v79, —.

2. Dean, H. R., *J. Hyg.*, 1912, v12, 259.

3. Hill, B. M., and Osler, A. G., *J. Immunol.*, 1955, v75, 146.

4. Morton, J. I., and Deutsch, H. F., *Arch. Biochem. and Biophys.*, 1956, v64, 26.

5. Weigle, W. O., and Maurer, P. H., *J. Immunol.*, 1957, v79,

6. Kekwick, R. A., and Cannan, R. K., *Biochem. J.*, 1936, v36, 227.

7. Talmage, D. W., Dixon, F. J., Bukantz, S. C., and Dammin, G. J., *J. Immunol.*, 1951, v67, 243.

8. Heidelberger, M., and Kendall, F. E., *J. Exp. Med.*, 1935, v62, 697.

9. Bier, O. G., Leyton, G., Mayer, M. M., and Heidelberger, M., *ibid.*, 1945, v81, 449.

10. Kabat, E. A., and Mayer M. M., *Experimental Immunochemistry*, 1948, C. C. Thomas, Springfield, Ill.

11. Bukantz, S. C., Rein, C. R., and Kent, J. F., *J. Lab. Clin. Med.*, 1946, v31, 394.

12. Markham, R., *Biochem. J.*, 1942, v36, 790.

13. Levine, L., Cowan, K. M., Osler, A. G., and Mayer, M. M., *J. Immunol.*, 1953, v71, 359.

14. ———, *ibid.*, 1953, v71, 367.

15. Levine, L., Osler, A. G., and Mayer, M. M., *ibid.*, 1953, v71, 374.

16. Mayer, M. M., Levine, L., Rapp, J. J., and Marucci, A. A., *ibid.*, 1954, v73, 443.

17. Mayer, M. M., and Levine, L., *ibid.*, 1954, v72, 516.

18. Levine, L., and Mayer, M. M., *ibid.*, 1954, v73, 426.

19. Laporte, R., Harde DeLooze, L., and Sillard, R., *Ann. Past. Inst.*, 1955, v89, 16.

20. Neff, J. C., and Becker, E. L., *J. Immunol.*, 1954, v73, 286.

21. Heidelberger, M., *J. Exp. Med.*, 1941, v73, 681.

22. Heidelberger, M., and Mayer, M. M., *Advances in Enzymology*, Interscience Publishers, Inc., N. Y., 1948, v8, 71.

23. Maurer, P. H., and Talmage, D. W., *J. Immunol.*, 1953, v70, 135.

24. Boyd, W. C., *ibid.*, 1940, v38, 143.

Received August 5, 1957. P.S.E.B.M., 1957, v96.

Hyperreactivity of *Coxiella burnetii* Infected Guinea Pigs to Subsequent Injections of Bacterial Endotoxins. (23482)

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Following Shwartzman's report(1) of a necropurpurogenic factor in meningococci, many workers noted that skin tissue could be prepared for the reaction by local infection with a number of microorganisms. Thus, *Bacillus anthracis*(2), *Mycobacterium tuberculosis*(3), *Haemophilus influenzae*(4), hemolytic streptococci(5), and even the virus of vaccinia(2,6) have been used to prepare animals for the local Shwartzman reaction. When animals with such skin infections were injected intravenously with a "provoking" dose of a culture filtrate from certain Gram-negative microorganisms capable of eliciting the Shwartzman reaction, hemorrhagic necrosis occurred at the infected skin site within a few hours. A generalized Shwartzman reaction has been produced in animals with systemic infections of *Vibrio cholera*(7), *Corynebacteria* and *Mycobacterium tuberculosis*(8), and Group A streptococci(9). The generalized Shwartzman reaction can also be consistently evoked by the intravenous injection of two doses of endotoxin from a number of Gram-negative bacteria. The most prominent pathological alteration seen in the classical generalized Shwartzman reaction has been reported to be bilateral renal cortical necrosis (5,10,11). While studying the interference of Brucella endotoxin on the course of a *Coxiella burnetii* infection in guinea pigs, a phenomenon was observed which appeared analogous to the generalized Shwartzman reaction. Guinea pigs infected with the rick-

ettsiae for 3 days developed an acute sensitivity when injected intracardially with *Brucella suis* endotoxin. Lipopolysaccharides extracted from two other Gram-negative bacteria could also be used to provoke animals similarly prepared. This paper presents data concerning the production of this hyperreactivity in guinea pigs infected with *C. burnetii*.

Materials and methods. *C. burnetii*, AD strain, was stored in a quick-frozen state as a 10% yolk sac suspension in heart infusion broth (Difco). Guinea pigs were infected by the intraperitoneal (IP) route with 1 ml of inoculum appropriately diluted in heart infusion broth. *B. suis* endotoxin was prepared essentially by the method of Boivin, as described by Kabat and Mayer(12). The IP LD_{50} of this preparation for 20 g albino mice was approximately 0.5 mg. A lipopolysaccharide extracted and purified from *Salmonella typhosa* was kindly supplied by Dr. M. Landy of the National Cancer Institute; a report from his laboratory has characterized this material(13). A similar preparation from *Escherichia coli* was obtained through the courtesy of Dr. D. Rowley of the Wright-Fleming Institute, London, England; this lipopolysaccharide also has been described in the literature(14). The provoking dose of endotoxin was invariably contained in 0.5 ml saline injected intracardially into etherized animals. A suspension of inactivated *C. burnetii* was used in certain studies as a substitute for the active rickettsiae. This material was prepared as follows: an emulsion containing 10^9 guinea pig IP ID_{50} of the rickettsiae per ml was treated with 0.2% formalin and incubated for 24 hours on a rotary shaker at 37°C. One ml of this preparation diluted 1:10 in heart infusion broth was injected intraperitoneally into guinea pigs as a preparative dose. In related studies, treatments with serum and antibiotic started on the day of infection and continued for 3 days thereafter were used to suppress the rickettsial infection. Guinea pigs received intramuscularly 1 mg tetracycline hydrochloride (Tetracyn, Chas. Pfizer and Co.), in 0.1 ml of distilled water twice daily. Pooled, filtered homologous antiserum was also employed which was obtained from guinea pigs that had survived for 28 days an infection produced with 10^8 guinea pig IP ID_{50} of *C. burnetii*. One ml of immune serum was injected intraperitoneally per animal per day.

TABLE I. Mortalities of *Coxiella burnetii* Infected Guinea Pigs Treated with Brucella Endotoxin. 10 animals/series.

Treatment schedule* (days)	No. of animals dead — Days after infection												
	1	2	3	4	8	11	12	13	14	15	16	17	18
−1						1	1	1			2	1	1
+3				7					1		1		
+8					1		1	5					
−1, +3, +8				9							1		
Untreated						1		1	2	1	2	1	
−1, +3, +8 (uninfected)													

* 100 μg Brucella endotoxin in 0.5 ml saline inj. intracardially per animal. Schedule of treatment is relative to the time of infection (0 day) with 10^3 guinea pig IP ID_{50} of *C. burnetii*.

Results. Table I presents the data of a typical experiment in which *B. suis* endotoxin was administered to guinea pigs infected with *C. burnetii*. After the rickettsial infection was established for 3 days, animals (treatments +3 and −1, +3, +8) were extremely sensitive to injections of 100 μg of the endotoxin, deaths occurring within 6-24 hours after treatment. Normal uninfected animals appeared refractory to multiple injections of Brucella endotoxin, and the susceptibility to *C. burnetii* of infected guinea pigs which survived treatment with Brucella endotoxin did not appear to change.

The comparative effects of varied amounts of 3 bacterial endotoxins on guinea pigs infected 3 days previously with *C. burnetii* are presented in Table II. While responses to *E. coli* and *S. typhosa* lipopolysaccharides were obtained with 10 μg amounts which were smaller than the 25 μg of Brucella endotoxin used, the relative purity of the 3 compounds must not be considered identical. The *E. coli* and *S. typhosa* lipopolysaccharides represented more highly purified materials than the Brucella endotoxin prepared for these studies.

Guinea pigs infected with *C. burnetii* demonstrated a maximum sensitivity to *E. coli*

TABLE II. Comparative Effects of Selected Bacterial Endotoxins on Guinea Pigs Infected with *Coxiella burnetii*.

Source of endotoxin	Endotoxin* (μg)	Mortalities,† dead/total
B. suis	100	4/4
	50	2/4
	25	1/4
	0	0/5
E. coli	50	5/5
	10	5/5
	1	2/5
S. typhosa	50	4/5
	10	2/4
	1	0/5

All animals infected 3 days previously with 10^8 guinea pig IP ID_{50} of *C. burnetii*.

* Contained in 0.5 ml saline inj. intracardially.

† All deaths occurred within 24 hr of endotoxin treatment. Uninfected animals survived injections of 100 μg of the bacterial endotoxins.

lipopolysaccharide when they were treated 48-72 hours post infection (Table III). The intensity of the response decreased when longer periods of time elapsed between the preparative rickettsial infection and the provoking treatment, although experimentally some animals were provoked as late as 8 days after infection.

An experiment designed to show the importance of the infecting dose of *C. burnetii* when animals were subsequently treated with Brucella endotoxin demonstrated that dosages as low as 10^6 guinea pig IP ID_{50} could be used to prepare animals suitably for this reaction. The responses of guinea pigs prepared with 10^6, 10^7, or 10^8 doses were identical when the provoking injection of 100 μg Brucella endotoxin was administered 3 days later. Those animals receiving 10^5 guinea pig IP ID_{50} or less did not respond to the provoking challenge of endotoxin. This appeared in agreement with Landy's observation that no clear-cut dose/effect relationship exists in the demonstration of this hyperreactive response (15).

Additional studies, summarized in Table IV, were performed to determine if an active infection of *C. burnetii* was required to prepare guinea pigs for this reaction. Guinea pigs which received 10^8 guinea pig IP ID_{50} of formalin inactivated rickettsiae were unaffected by a provoking dose of 100 μg Brucella endotoxin administered 3 days later. It was interesting to note also that when infected animals were treated daily with either homologous antiserum or Tetracyn for the 3-day interval between infection and the time of challenge they appeared quite resistant to provoking injections of Brucella endotoxin. The success of these therapeutic treatments was indicated by the fact that all treated guinea pigs survived the rickettsial infection which normally would have killed 70% of the animals.

Further experiments failed to demonstrate any increase of rickettsiae in either the blood

TABLE III. Significance of Time in Provoking the Hyperreactive Response in *Coxiella burnetii* Infected Guinea Pigs Treated with *Escherichia coli* Lipopolysaccharide. 5 animals/series.

Treatment schedule*	Mortalities,† dead/total
0 day	0/5
1	3/5
2	5/5
3	5/5
4	3/5
5	0/5

* 100 μg lipopolysaccharide in 0.5 ml saline inj. intracardially per animal after their infection on 0 day with 10^8 guinea pig IP ID_{50} of *C. burnetii*.

† All deaths occurred within 24 hr of treatment. No uninfected animals died which received lipopolysaccharide alone.

TABLE IV. Effects of Various Preparative Injections and Subsequent Treatments on the Hyperreactivity Response in Guinea Pigs Provoked with Brucella Endotoxin.

Preparative inj.*	Treatment (for 3 days)	Provoking dose (intracardially)	Mortalities,§ dead/total
C. burnetii	None	100 μg *B. suis* endotoxin	5/5
	Homologous antiserum†	*Idem*	1/5
	Tetracyn‡	"	0/5
	None	None	0/5
Formalin inactivated *C. burnetii*	None	100 μg *B. suis* endotoxin	0/5
	"	None	0/5

* 10^8 guinea pig IP ID_{50} inj. IP/animal. † 1 ml IP/animal/day. ‡ 1 mg intramusc. twice daily/animal. § All deaths occurred within 24 hr of endotoxin inj.

or the spleen of infected guinea pigs after treatment with Brucella endotoxin. Because of this, and because deaths occurred so quickly—in many cases as early as 6 hours after treatment with bacterial endotoxins—this phenomenon was considered to be a hyperreactive response, perhaps analogous to the generalized Shwartzman reaction.

In an effort to determine if the observed hyperreactivity was a manifestation of the generalized Shwartzman reaction, observations were made of the gross pathology of 30 necropsied guinea pigs which had succumbed to provoking doses of bacterial endotoxins 3 days after their infection with *C. burnetii*. Hyperemia of the adrenals and a variable amount of congestion of the mesenteric vessels were consistent findings, together with changes in the spleen typical of an early rickettsial infection. In those infected animals surviving the provoking dose of endotoxin some indication of adrenal hyperemia was present although the reaction was not as marked. Congestion of the mesenteric vessels of the animals in this group was extremely variable, both in degree and in occurrence.

The following organs were routinely removed for histological studies: liver, spleen, heart, lungs, kidneys, adrenals, mesenteric lymph nodes and sections of the intestine. Microscopic examination of tissues from 7 infected animals that died of the provoking challenge disclosed hemorrhage, hyperemia, some leucocytic infiltration into the adrenal cortex, and hyperemia of the vessels in the adrenal medulla. It should be noted that one animal failed to develop this pathology. Indications of an early granulomatous-like reaction were found in the spleen and liver of all the animals in this group. Tissues from 8 of 11 infected animals which survived the provoking dose of endotoxin showed a similar pathological response, but the changes were less prominent, and hemorrhage was occasionally absent. The remaining 3 guinea pigs in this group showed only microscopic evidence of the rickettsial infection. There was no indication of bilateral renal cortical necrosis in any of the animals examined.

In a limited number of uninfected guinea pigs treated with bacterial endotoxin no gross or microscopic pathology was noted.

In discussing the significance of these findings, two observations are worthy of note. The first is the consistent presence of adrenal pathology in *C. burnetii* infected guinea pigs subsequently treated with endotoxin preparations. The second is the complete absence of bilateral renal cortical necrosis in this same experimental group. Gronchi and Carnielli (16) have reported histological studies on the adrenals of guinea pigs which received 2 intracardial injections of a broth filtrate of *E. coli*. With this regimen which would elicit the generalized Shwartzman reaction they noted essentially the same pathology as described here. As an explanation for the absence of renal cortical necrosis reported to be characteristic of the generalized Shwartzman reaction, it is possible that although this lesion is a constant finding in the rabbit, the usual laboratory animal for such studies, it does not occur in guinea pigs.

Since the hyperreactivity elicited in *C. burnetii* infected guinea pigs by subsequent injections of bacterial endotoxins conforms generally in character to the generalized Shwartzman reaction, and because the pathology noted is in keeping with observations of others who have studied this reaction in guinea pigs, it is suggested that the hyperreactivity noted here is a manifestation of the generalized Shwartzman reaction.

Summary. Guinea pigs infected for 3 days with *C. burnetii* demonstrated a hyperreactivity to injections of bacterial endotoxins, with deaths occurring 6-24 hours after treatment. The preparative infectious dose of *C. burnetii* could be as low as 10^6 guinea pig IP ID_{50}. The reaction has been provoked with 25 μg of Brucella endotoxin while 10 μg of purer preparations from *E. coli* and *S. typhosa* have produced a similar response. An optimal period of reactivity existed from 48-72 hours post infection. Formalin inactivated rickettsiae did not prepare animals for this reaction and infected guinea pigs treated with either rickettsial antiserum or Tetracyn could not be provoked by subsequent injections of endotoxin. The gross and microscopic studies

of selected tissues taken from hyperreactive animals consistently revealed hyperemia and hemorrhage of the adrenals. The described hyperreactivity appears to be a manifestation of the generalized Shwartzman reaction.

1. Shwartzman, G., PROC. SOC. EXP. BIOL. AND MED., 1929, v26, 207.

2. Gratia, A., and Linz, R., *Compt. rend. Soc. biol.,* 1931, v107, 1579.

3. Debonera, G., Tzortzakis, N., and Falcketti, E., *ibid.,* 1932, v109, 24.

4. Witebsky, E., and Salm, H., *J. Exp. Med.,* 1937, v65, 43.

5. Shwartzman, G., *Phenomenon of Local Tissue Reactivity,* Paul B. Hoeber, Inc., N. Y., 1937.

6. Koplik, L. H., *Am. J. Path.,* 1935, v11, 842.

7. Sanarelli, G., *Ann. Inst. Pasteur,* 1924, v38, 11.

8. Bordet, P., *ibid.,* 1936, v56, 325.

9. Thomas, L., Denny, F. W., and Floyd, Joan, *J. Exp. Med.,* 1953, v97, 751.

10. Black-Schaffer, B., Hiebert, T. G., and Kerby, G. P., *Arch. Path.,* 1947, v43, 28.

11. Thomas, L., and Good, R. A., *J. Exp. Med.,* 1952, v96, 605.

12. Kabat, E. A., and Mayer, M. M., *Experimental Immunochemistry,* 1st Edit., Charles C. Thomas, Springfield, Ill., 1948, p514.

13. Webster, M. E., Sagin, J. F., Landy, M., and Johnson, A. G., *J. Immunol.,* 1955, v74, 455.

14. Rowley, D., *Brit. J. Exp. Path.,* 1956, v37, 223.

15. Landy, M., and Johnson, A. G., PROC. SOC. EXP. BIOL. AND MED., 1955, v90, 57.

16. Gronchi, V., and Carnielli, P., *Atti Soc. Med.-Chir. Padova,* 1934, v12, 433. (Biol. Abstr., 1936, v10, No. 6109).

Received August 5, 1957. P.S.E.B.M., 1957, v96.

Action of Sodium Desoxycholate on Arthropod-Borne Viruses. (23483)

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As a by-product of studies on the course of infection of monkeys with virulent yellow fever virus(1), it was discovered that bile from normal rhesus monkeys had a marked virucidal action on the virus. Bile in a dilution of 1:50 was shown to inactivate from 3.0 to 5.0 logs of several strains of yellow fever. In extending the experiments to determine the action of bile on other agents, it was discovered that viruses could be divided into two groups—one resistant and the other susceptible to the action of bile. Viruses which were resistant were the Lansing and MEF_1 strains of poliomyelitis, the GD VII strain of mouse encephalomyelitis and the Mengo strain of encephalo-myocarditis. Agents found to be susceptible were yellow fever, Uganda S, Ntaya, West Nile, dengue 1, dengue 2, St. Louis, Japanese B, Ilheus, EEE, Bwamba, Bunyamwera and Anopheles A. The method of determining the degree of virus inactivation was to make parallel titrations of the virus in the presence or absence of a 1:50 dilution of bile. A summary of such an experiment appears in Table I. The diluent used in these experiments was either 10% normal monkey serum in saline or a 0.2 solution of bovine albumen (bpa) in saline.

In the course of these experiments it was found that samples of bile from different normal rhesus monkeys varied in their capacity to inactivate a bile susceptible virus. This observation led to the search for a more clearly defined chemical substance. The use of sodium desoxycholate (DCA) was a logical choice as this salt had been used extensively in the study of the bile solubility of the pneumococcus. The results of a preliminary experiment to determine the action of DCA are shown in Table II. In this experiment, the action of DCA in two concentrations (1:1000 and 1:10,000) was tested on yellow fever virus and the higher concentration on GD VII. In addition, the yellow fever virus was titrated in the presence of a 1:50 dilution of bile. It will be observed that the 1:1000 dilution of DCA inactivated the yellow fever virus to approximately the same extent as the 1:50 dilution of bile, whereas this concentration of DCA had no action on the GD VII virus. The

TABLE I. Action of Bilė on Yellow Fever, Bwamba, GD VII Strain of Mouse Encephalomyelitis and MEF_1 Strain of Poliomyelitis.

Dilution of virus	Yellow fever		Bwamba		GD VII		MEF_1	
	Bile 1:50	Control	Bile 1:50	Control	Bile 1:50	Control	Bile	Control
10^{-1}					6/ 6	12/12	11/11	12/12
10^{-2}	0/8	10/10	0/12	12/12	12/12	"	"	"
10^{-3}	0/6	"	"	"	"	"	12/12	11/11
10^{-4}	0/7	9/10	"	"	"	"	"	8/12
10^{-5}	0/8	1/10	"	5/12	"	"	8/11	2/12
10^{-6}	"	0/10	"	0/12	"	11/12	4/12	1/12
10^{-7}			"	1/12	12/13	1/11		
Titer	$<10^{-2}$	$10^{-4.5}$	$<10^{-2}$	$10^{-4.9}$	$>10^{-7.4}$	$10^{-6.5}$	$10^{-5.6}$	$10^{-4.4}$

higher dilution of DCA had no effect on the yellow fever virus. In these experiments, an equal portion of the virus dilution was mixed with the solution of DCA or bile, and, hence, the final dilution of these was 2-fold higher. In further work, to determine the action of DCA on viruses, this salt was used in a dilution of 1:500, so that when mixed with an equal quantity of the virus dilution, the concentration in the mixture was 1:1000. Certain factors were of vital importance to obtain clearcut results. It was found that the DCA at times failed to inactivate an agent which had previously been shown to be susceptible. In these instances, it could be readily shown that the failure of the DCA to inactivate the virus was associated with the method of preparing the virus suspension. In every case of DCA failure, the virus had been prepared by light centrifugation. When the same virus suspension was submitted to an hour's centrifugation at 10,000 rpm in an angle centrifuge, it was readily inactivated by the DCA. This method of preparation has been adopted as a routine. Another factor, which has been shown to be of importance, is the concentration of protein in the mixtures. It was found that the action of DCA could be completely inhibited by the presence of high concentrations of protein in the mixtures. When a 1:500 dilution of DCA in 0.75% bpa was mixed with virus dilutions made in 7.5% bpa, no inactivation occurred. Similarly, the action of DCA in 10% normal monkey serum was completely inhibited when mixed with virus dilutions made in undiluted normal monkey serum. Consequently, as a standard procedure, centrifugation at 10,000 rpm and a 0.75% bpa diluent has been adopted. The rate of inactivation of a virus by DCA has not been determined, but it has been found that incubating the mixtures for 1 hour at 37°C has given satisfactory results.

TABLE II. Action of Sodium Desoxycholate (DCA) on Yellow Fever and GD VII Strain of Mouse Encephalomyelitis.

Virus dilution	Yellow fever				GD VII
	DCA 1:1000	DCA 1:10,000	Bile 1:50	Control	DCA 1:1000
10^{-2}	6/6	6/6	3/6	6/6	
10^{-3}	1/6	"	0/6	"	6/6
10^{-4}	"	"	"	"	"
10^{-5}	0/6	"	"	"	"
10^{-6}	"	"	"	"	"
10^{-7}	"	2/6	"	2/6	4/6
10^{-8}	"	1/5	"	1/6	3/6
10^{-9}					0/6
Titer	$10^{-2.8}$	$10^{-6.9}$	10^{-2}	$10^{-6.9}$	$10^{-7.7}$

The following arbor viruses were inactivated by DCA. The figures in parentheses indicate logs of virus inactivated. *Group A.* EEE (3.0, 5.5), WEE (6.0), VEE (3.0, 4.7), Semliki Forest (4.0, 5.4), Sindbis (2.6, 4.1, 5.0). *Group B.* Yellow fever (4.2, 5.1, 3.1, 4.5), Japanese B (5.0), St. Louis (3.5, 4.1, 3.8), Ilheus (3.8, 4.8), West Nile (5.8), Uganda S (2.8, 3.3, 3.9), Zika (3.7), dengue 1 (3.1), dengue 2 (4.6, 3.7), Russian spring-summer (5.0), Ntaya (2.8). *Miscellaneous.* Anopheles A (3.6), Anopheles B (2.5), Wyeomyia (3.6), California virus of Hammon and Reeves (2.1), Bunyamwera (3.5), Bwamba (3.0), Rift Valley fever (2.0), the Sicilian (2.8) and the Naples strains of Sandfly fever (3.0). In addition, several recently isolated agents thought to be arthropod-borne, at present under study and apparently new to science, have all been shown to be inactivated by DCA.

Results. The following strains of virus proved to be resistant to the action of DCA. The negative sign before the figures in parentheses indicates that the virus gave a higher titer in the presence of DCA than in the control. GD VII strain of mouse encephalitis (0.0, –0.8, –0.6, –0.6), FA strain of mouse encephalomyelitis (–0.3), Coxsackie virus (0.3, –0.8, –0.3), MEF_1 strain of human poliomyelitis (–1.0, –0.5), Mengo strain of EMC (0.1, 0, –1.0, 0). It is of interest to note that the agents in the DCA resistant group, as a rule, give a slightly higher titer in the presence of DCA than they do in the controls.

The finding that viruses could be divided into bile salt resistant and susceptible groups has been of great value as a preliminary step in identification. In the attempted isolation of viruses by the inoculation of mice, on numerous occasions, strains of encephalomyelitis virus of mice have been accidentally obtained. Being resistant to the action of DCA, this virus can be readily distinguished from the arbor viruses. In recent years, more and more arbor viruses (*e.g.*, Sindbis) pathogenic for infant mice only have been isolated. These can be readily distinguished from the Coxsackie group because the former are susceptible and the latter resistant to the action of DCA. Of unusual interest is the finding that the Mengo strain of EMC is resistant. This agent has been isolated on several occasions from mosquitoes and was for some time considered to be arthropod-borne. However, extensive experiments have shown that this agent does not multiply in the mosquito and no successful experimental transmission by bite has been obtained. Infection with this agent leads to a viremia and, consequently, it is not surprising that at times mosquitoes will be caught in nature apparently infected with this agent. In many of its characteristics the Mengo virus is closely related to the polio group of agents. It is of interest here to recall that Kerr(2), by means of the complement fixation test, has shown an immunological relationship between the Mengo virus and the GD VII strain of mouse encephalomyelitis.

Susceptibility to DCA is not an exclusive property of the arbor group of viruses. Agents such as influenza A and lymphocyte choriomeningitis are readily inactivated.

Summary. All arthropod-borne viruses at present under study in the Rockefeller Foundation Virus Laboratories, New York, have been shown to be readily inactivated by a 1:1000 dilution of sodium desoxycholate. The only viruses discovered to date which are resistant to the action of this bile salt are strains of human poliomyelitis, mouse encephalomyelitis, Coxsackie and encephalomyocarditis viruses.

1. Theiler, M., *"The Virus" in Yellow Fever,* edited by George K. Strode, 1951, McGraw-Hill Book Co., Inc.

2. Kerr, J. A., *J. Immunol.,* 1952, v68, 461.

Received August 6, 1957. P.S.E.B.M., 1957, v96.

Maze Performance, Emotionality, Audiogenic Seizure Susceptibility in Rats and Mice Treated with 2-Dimethylaminoethanol (23484)

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The intimate relationship of acetylcholine to the transmission of impulses in the nervous system has led to the expectation that changes in the metabolism of this substance must have pronounced effects upon various facets of behavior. This expectation has given rise to a number of investigations with confirming results. For example Ginsburg and Hovda(1) and Ginsburg and Huth(2) have described a negative relation between susceptibility to audiogenic seizures and several parasympathomimetic substances. Acetylcholine and

eserine reduced the incidence and fatality of such attacks. Prostigmine also reduced the number of fatalities. In addition they report a central decrease and peripheral increase of free acetylcholine following seizures in a very sensitive strain of mice. Rinaldi and Himwich(3) have stated that acetylcholine and di-isopropyl-fluorophosphate (D.F.P.) stimulate the "mesodiencephalic activating system" and that D.F.P. produces signs of anxiety and restlessness in human subjects. Krech *et al.* (4) describe reliably higher cholinesterase activity in the visual and somesthetic cortex in strains of rats which had been bred to select predominantly visual cues in maze learning. Such animals learned mazes more quickly than those depending primarily upon kinesthetic information. Dimethylaminoethanol (DMAE) has been studied by Pfeiffer *et al.* (5) as a possible tertiary amine precursor of acetylcholine. If DMAE acts as a precursor it might be expected to produce behavioral alterations similar to those attributed to acetylcholine. The studies reported here form a part of a larger program initiated by Pfeiffer to investigate the pharmacology, physiology, and behavioral effects of substances which may produce rather long lasting muscarinic stimulation of the brain and which may possess ameliorative properties in mental disease. In all, 7 experiments involving 2 genera, were performed. Three were concerned with the possible effects of DMAE on maze performance, 3 with emotionality, and one with seizure susceptibility.

1. *T-Maze performance in mice.* Two groups of male Swiss Albino mice (average weight 25 g) were trained to run a 3-unit elevated T-Maze learned one segment at a time. The length/unit was 12 inches and the paths were 1.5 inches wide. Learning was defined as achieving a proportion of correct responses significant at the one per cent confidence level. This criterion differs from the usual one involving a specific proportion of correct responses on an arbitrarily designated number of trials. It was used to minimize overlearning. The incentive for learning was food preceded by 23 hours' fasting. The experimental group received 1.0% DMAE administered intravenously; the control group received no pharmacological treatment. Performance in this as well as all subsequent experiments was evaluated by non-parametric statistical procedures. Two group comparisons were evaluated by means of the Mann-Whitney U test: three or more groups by the Kruskal-Wallis H test.

The *results* indicated that performance was superior in the control group (median trials to criterion: 30 *vs.* 35). This difference was significant at the .01 level ($U = 28$, $m/n = 12/11$). The experimental animals were meanwhile noted to be hyperactive and difficult to handle. The significance of this for the maze data will be discussed later.

2. *Serial-maze performance in mice.* Several weeks after Exp. 1 the 2 groups described above were tested on a 4-unit enclosed serial maze (LRLR). The alleys were 3.5 in. wide and 16 in. long. Criterion of learning was a conventional two consecutive errorless trials without retracing. For this experiment the drug was administered as 0.05% in drinking water to which the experimental group had *ad libitum* access. The mean daily intake of DMAE was 2.25 mg. This time no difference in trials to criterion was observed (experimental median = 22.4; control median = 20.6, $U = 58$, $m/n = 12/11$, $P > .05$).

3. *Emotionality in mice.* Two tests of emotionality were run on the mice which had learned the maze problems. These tests were run immediately after learning had occurred in each instance. In the Stone stove-pipe maze (an L-shaped enclosed opaque alley with long arm 36 inches, base 12 inches, and short arm 24 inches) emotionality is indicated by the time required for the mouse to enter a brightly illuminated goal box. In the Anderson water-wading test, emotionality is indicated by the number of boli passed in a 5-minute period when an animal is placed in an enclosed opaque chamber in water one cm deep at approximately 25°C. Each animal was tested 3 times on each test, individual tests being separated by 48 hours. After Exp. 1 experimentals were more emotional than the controls on both tests. (Median time in stove-

pipe: 270 sec. *vs.* 110 sec., U = 13, m/n = 12/11, P<.001; water wading median number of boli = 2 *vs.* 1, U = 29, m/n = 12/11, P<.025). After Exp. 2 involving oral administration of DMAE, no significant differences were found between the groups.

4. *Serial-maze performance in rats.* Three groups of 4-month-old male and female albino rats (total N = 28), derived from Wistar stock, were trained in a 4-unit enclosed serial maze. They were trained on the first unit to criterion, then on Units 1 and 2, Units 1, 2, and 3, and finally on Units 1, 2, 3, and 4 to criterion. The criterion was the proportion of correct responses significant at the one per cent significance level. One group served as the control (C), the other 2 as experimental groups. These latter were placed on the drug regimen shortly after weaning. One (E_1) received 0.1% solution of DMAE in water for *ad libitum* oral intake, the other (E_2) a 1% solution. Both E Groups were continued on the drug throughout the maze-learning period. This was approximately 109 days for E_1 and 189 days for E_2. The average daily fluid intake for C, E_1 and E_2 was 20.1 ml, 18.0 ml, and 16.0 ml respectively.

Performance in the maze problem was measured by the following scores: (a) median trials to criterion, (b) median total errors to criterion, (c) median total time to criterion, (d) median errors per trial, (e) median time per trial. No significant differences were found between the control group and E_1 (the 0.1% dose group); E_2, however, gave, on the average, poorer performance than either C or E_1 on trials to criterion, total errors and errors per trial. No difference in performance times was noted. The median performance scores were as follows: median trials to criterion: C = 125, E_1 = 130. E_2 = 165; median total errors: C = 33, E_1 = 32, E_2 = 90; median errors per trial: C = .28, E_1 = .35, E_2 = .51; median total time: C = 1743″, E_1 = 3647″, E_2 = 5843″; median time per trial: C = 15″, E_1 = 24″, E_2 = 30″. The animals in group E_2 were observed to have frequent spontaneous clonic convulsive attacks, often rather severe. These seizures occurred both in the home cage and in the maze and probably account for the poorer performance of E_2.

5. *Emotionality in rats.* The rats used in Exp. 4 were given emotionality tests after completion of maze training. Again, 2 tests were used: the Stone stovepipe maze, and the Anderson water-wading test. No significant differences were found between C and E on either test, although the medians rank from low to high emotionality in the expected direction (C, E_1, E_2). The median values for the several groups were as follows: water-wading: C = 4. E_1 = 3.5, E_2 = 5; Stone test: C = 51, E_1 = 81, E_2 = 300.

6. *Susceptibility to audiogenic seizures in mice.* Four groups of mice, one control and 3 experimental (total N = 20), were tested for susceptibility to audiogenic convulsions before and after administration of DMAE. They were all males of an inbred strain (A strain) with an average weight of 30 g on the day of first test. Tests consisted of 2-minute exposures to noise (average 101 db, range 90 to 104 db) on alternate days. Prior to administering DMAE to the experimentals, all animals were given a sequence of 5 tests. The 3 experimental groups were then placed on *ad libitum* oral intake of 0.01%, 0.10%, and 1.0% solutions of the drug respectively. They were maintained on this until the experiment was completed. The average daily intake was 0.6, 5.4, and 40.0 mg respectively. Two weeks after onset of drug administration, a second series of 5 tests was begun. Seizure susceptibility was ascertained by the following measures: (a) frequency index: this is the proportion of tests on which a running or epileptiform attack was observed; (b) intensity index: this is a weighted score derived from the total number of running and convulsive attacks; (c) latency of first running attack; (d) latency of first convulsion; (e) duration of running prior to convulsion; (f) total time in convulsion. After the groups were matched, the changes in susceptibility from the first to the second test sequence were compared. No significant differences were found between the control group and the experimentals on the frequency index, the latency of first epileptoid fit, or the total con-

vulsion time. Significant differences ($P = .02$ or less) were found between experimentals and controls on the intensity index, the latency of first running attack and the duration of running prior to the epileptoid sequence. At the end of sequence 2 the experimentals were almost without exception more susceptible than their matched controls. Meanwhile no significant differences were found among the three experimental groups. The controls showed the commonly observed decrease in susceptibility with increasing age and repeated tests. In contrast, the experimentals retained their initial sensitivity or showed a slight increase in sensitivity. Thus on the second sequence of tests, the experimentals showed more intense attacks, went into the running attacks sooner, and ran for a shorter period before displaying the epileptiform seizures.

Discussion. Animals receiving DMAE tended to show increased emotionality as indicated by the Stone and Anderson tests. This finding is in agreement with the observations of Jenny,* Grob, *et al.*(6), and Killam and Killam.* Jenny reported that rats given daily doses of DMAE became more irritable, difficult to handle, and exhibited markedly increased muscular tension as well as spontaneous seizures. Grob showed that the administration of D.F.P. produced symptoms of restlessness and anxiety in human subjects, while Killam and Killam found that DMAE increased the amount of cortical low voltage fast activity and reduced the threshold at which reticular stimulation produced a cortical EEG pattern characteristic of the arousal response.

These findings indicate a correlation between the increased emotionality and irritability produced by DMAE in our animals and stimulation of the brain stem reticular formation. Our finding of increased sensitivity to audiogenic seizures might also be expected in light of the above mentioned reports.

The performance decrements exhibited by rats maintained on daily 1% solutions of DMAE is interpreted as secondary to increased emotionality and to the occurrence of spontaneous epileptiform seizures during testing. We have no evidence to indicate that learning capacity *per se* is adversely affected. Our data indicate that when hyper-emotionality disappeared the animals exhibited no performance decrement.

Summary. Rats and mice when given 0.1% and 1.0% solutions of 2-dimethylaminoethanol (DMAE) to drink for prolonged periods showed either a decrement or no improvement in maze performance. They exhibited increased emotionality as measured by the Stone and Anderson tests. Rats given 160 mg/day were subject to frequent spontaneous epileptiform seizures. Inbred (A-strain) mice when tested for susceptibility to audiogenic seizures showed more intense attacks with shorter latent periods while on DMAE. The decremental effect of DMAE upon maze performance was interpreted as a secondary effect of increased emotionality and/or epileptiform seizures. The facilitatory effect upon audiogenic seizure susceptibility is interpreted as due probably to stimulation of the brain stem reticular formation.

1. Ginsburg, B. E., and Hovda, R. B., *Anat. Rec.,* 1947, v99, 621.

2. Ginsburg, B. E., and Huth, E., *Genetics,* 1947, v32, 87.

3. Rinaldi, F., and Himwich, H. E., *Dis. Nerv. System,* 1955, v16, 133.

4. Krech, D., Rosenzweig, M. R., Bennett, E. L., and Krueckel, B., *Science,* 1954, v120, 994.

5. Pfeiffer, C. C., Jenney, E. H., Gallagher, W., Smith R. P., Bevan, W., Miller, I., Killam, K. F., Killiam, E. K., and Blackmore, W., *Science,* 1957, v126, 610.

6. Grob, D., Harvey, A. M., Longworthy, O. R., and Lilienthal, J. L., *Bull. Johns Hopkins Hosp.,* 1947, v81, 257.

* Personal communication.

Received August 12, 1957. P.S.E.B.M., 1957, v96.

Resistance of Respiratory Ciliated Epithelium to Action of Polio and Adeno Viruses *in vitro*. (23485)

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It has been observed that a tissue entirely resistant to a viral infection *in vivo* may become highly sensitive to the same infection after explantation *in vitro*. This phenomenon is well illustrated by the behavior of the kidney of the monkey in which no multiplication of poliovirus occurs when the agent is inoculated directly in the organ *in vivo* but which supports a rapid multiplication of the same virus when separated from the organism and maintained in tissue culture(1). Since the dominant feature of the evolution of *in vitro* explanted tissues is their histomorphological disorganization and their functional dedifferentiation, we undertook to investigate with certain particular tissues in which the cellular differentiation may be easily recognized *in vitro*, the relation between the degree of differentiation and the sensitivity to viruses. The ciliated epithelium of the respiratory mucosa appeared to us as a particularly suitable material for this study. We used in this work *in vitro* cultures of human (adult and embryo) and simian (adult) tissues of the bronchial and tracheal lining. To some of these cultures were added other cellular implants remarkable for their dedifferentiated, astructural growth, such as kidney epithelium and the human carcinoma HeLa cell strain(2). Type I poliovirus and type I adenovirus were used as viral agents.

Material and methods. Tissue cultures. All of our chicken plasma embedded cultures were prepared on 12 x 32 mm cover slips in 16 x 160 mm flattened tubes(3). Fragments of human bronchial lining tissue were excised by biopsy at the intersection of the bronchus of the right middle lobe and the main bronchus in adults of both sexes with a normal bronchoscopic aspect of their mucosa. The fragments (1-2 mm size) were directly transferred into cultures in an entirely heterologous medium containing 0.5% lactalbumin hydrolysate in Earle's solution supplemented with 0.1% yeast extract, 5% bovine embryo extract and 10 to 15% horse serum. The same medium was also used for the cultures of human embryo and monkey tissues (*c.f.* later). Human tracheal tissue was obtained from a premature newborn child (5½ months *in utero*) 5 hours after death. 0.5 to 1.0 mm thick fragments were cut perpendicularly to the long axis of the trachea and placed for culture with their cut face applied to the cover slips. This disposition is essential for the observation of the ciliary movement. Fragments of monkey trachea were prepared in the same way. The sensitivity to poliomyelitis virus of the *in vitro* cultivated tissues from the African monkeys *Erythrocebus patas* used in this work had been previously demonstrated(4) and confirmed(5). In the present work the virus sensitivity was tested additionally for each individual monkey on cultures prepared with one of its kidneys previously removed by nephrectomy. It appeared that the sensitivity of *Erythrocebus* kidney cell cultures to poliovirus was essentially similar to that of the Asiatic Cynomolgus kidney cultures,* and their sensitivity to the type I adenovirus was comparable to that of the HeLa strain cultures. Further, plasma imbedded or trypsin dispersed cell cultures of human embryo kidneys, monkey kidneys and the HeLa strain were employed separately, or associated in the same tubes with tissues of the respiratory tract. Observation of the cultures was performed either directly in the tubes or after mounting the culture bearing cover slips in special perfusion chambers(6). For more detailed observation the cultures were fixed with Bouin fixative and the growth area stained with hematoxylin-eosin directly on the culture cover slip. The tissue fragments after removal were sectioned for

* Obtained from Microbiological Associates.

slide preparations by the usual histological technics. *Virus.* The Mahoney poliovirus type I and the adenovirus Ad. 71 type I strain† were used. The virus titrations and neutralization tests were performed in the usual way in tube cultures of trypsinized monkey kidney cells for poliovirus and in similar cultures of the HeLa strain for the adenovirus.

Results. 1. *Human bronchial tissue and poliovirus.* Of the total number of 8 biopsied patients (31 to 62 years old) 6 furnished viable tissue material showing after explantation *in vitro,* ciliary movement and cell proliferation.

The cilia were particularly active at the edges of the fragments where they could be observed immediately after explantation and over a period of two to three weeks. With isolated cells or cell agglomerations this vibratile activity ceased after 5 to 6 days.

Growth, beginning on the 3rd or 4th days, was predominantly epithelial. Isolated spots of ciliated cells were observed in the growth area close to the fragments until the 22nd day after explantation.

HeLa cells implanted in the human bronchial cultures proliferated but did not visibly disturb the development and the activity of these cultures during a period of observation of 15-20 days.

When 10^5 $I.D._{50}$ of poliovirus were introduced into this type of, 5 to 7 days old, mixed cultures, a complete destruction of the HeLa cells occurred after 48 hours whereas in the growth area of the bronchial epithelium the cytopathogenic process was progressing only slowly from the periphery of the growth area towards the edges of the fragments; healthy areas could be seen in the vicinity of the fragments as late as 10 days after introduction of the virus. High degree of resistance of the ciliated cells to virus action could be shown in all 6 series of human bronchial cultures. Distinct activity of the cilia was observed, 5, 10, and in one series, 19 days after introduction of the virus. This activity was particularly tenacious on the explants but was also observed in the proximate growth area. During the observation period the virus titers were constantly high. This may be illustrated by the example of one series (bronchial cultures associated with HeLa cells) in which the viral titers were $10^{6.8}$ 24 h., $10^{7.5}$ 48 h. and $10^{6.8}$ $I.D._{50}$ per ml 5 days after infection.

2. *Human embryo trachea cultures and poliovirus.* Explants of human embryo trachea have shown a mixed, chiefly epithelial growth which started from the 3-4th day and became quite extensive after one week. The ciliary movement was intense and constant on the interior borders of the fragments.

When 10^5 $I.D._{50}$ of poliovirus were added to the mixed trachea-kidney cultures whether on the 3rd or on the 7th day, the growth areas were entirely destroyed after 48 hours around the kidney and after 3 days around the trachea fragments. Nevertheless uninterrupted ciliary activity could be observed 4 days after introduction of virus when it was present in a titer of $10^7 ID_{50}$ per ml.

In sections, the lining epithelial sheet appeared flattened but composed of entirely normal cells, the underlying connective tissue was necrotic while the glandular epithelium and the cartilage seemed normal.

3. *Monkey trachea cultures and poliovirus.* The ciliary activity of the explanted monkey tracheal fragments was evident during a period of 45 days *in vitro.* In the growth area, predominantly epithelial islands of ciliated cells were visible until the 20th day. Monkey kidney tissue fragments or HeLa cells if added to these cultures were growing abundantly, apparently without disturbing the tracheal cultures. 10^5 ID_{50} of virus added to these combined cultures induced after 48 hours a complete destruction of the growing HeLa and monkey kidney cells. This was followed by a rise in virus titers to 10^6-10^7 ID_{50} per ml. Titrations on the 5th day still gave titers of $10^{6.5}$ and on the 11th day of $10^{5.8}$ per ml.

These high virus concentrations did not appear to influence the vibratile activity of the ciliary cells which was clearly seen as long as 10 days after the infection. During this period the growth area of the tracheal epithelium outside the fragments was progressively attacked by the virus but uninjured narrow epithelial sheets with beating cilia

† Received from Dr. R. Huebner, (N.I.H.)

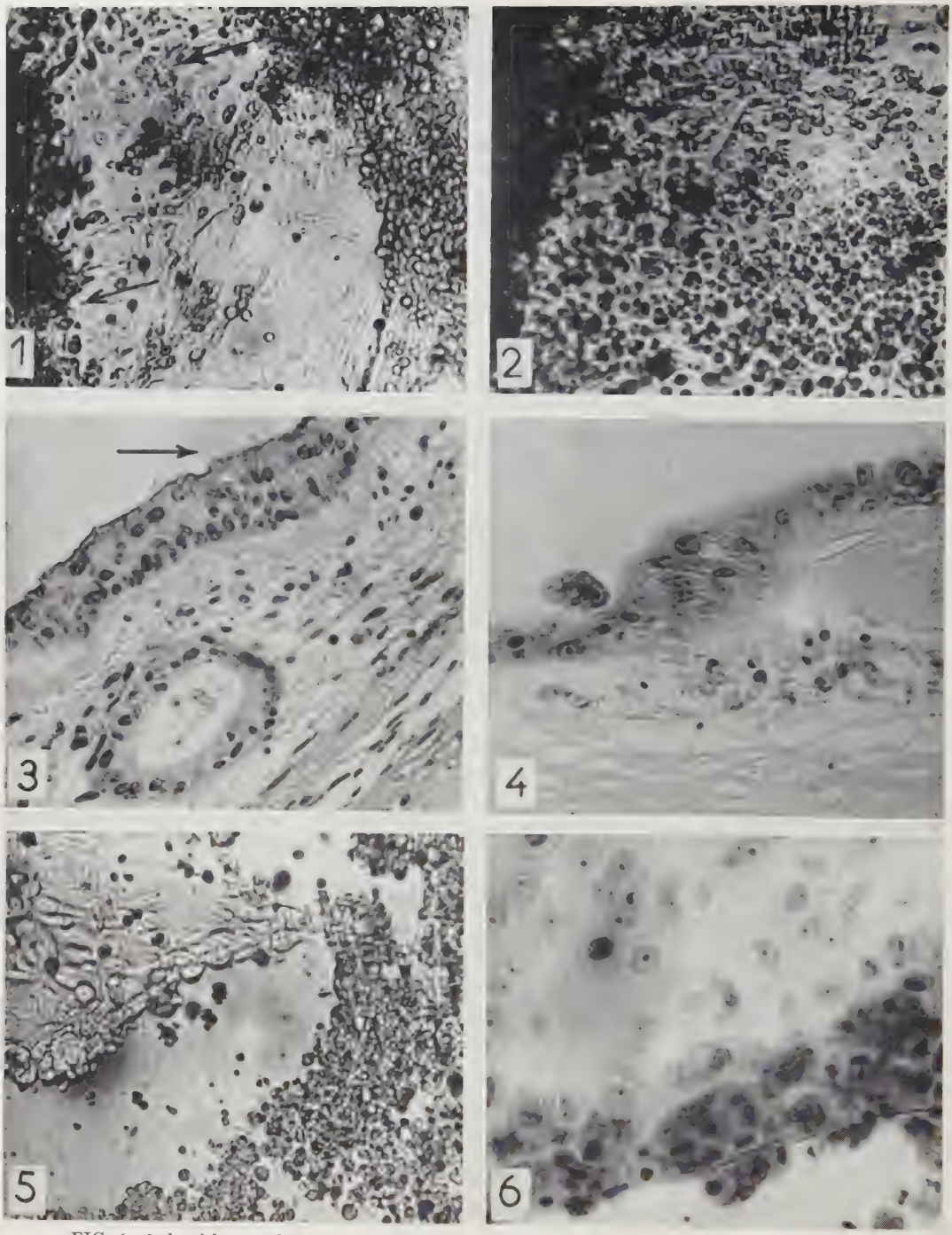

FIG. 1. 9 days' human bronchial culture; 3 days with poliovirus. Active ciliary cells (arrows).
FIG. 2. 9 days' monkey kidney culture; 3 days with poliovirus.
FIG. 3. 9 days' monkey tracheal culture; 6 days with poliovirus. Nearly normal glandular and lining epithelium with active cilia. Connective tissue cells destroyed.
FIG. 4. 11 days' monkey tracheal culture; 8 days with poliovirus. Normal lining epithelium.
FIG. 5. 14 days' monkey tracheal culture associated with HeLa cells; 4 days with adenovirus. HeLa cells (at right) completely destroyed.
FIG. 6. 16 days' monkey tracheal culture; 6 days with adenovirus. Virus lesions on the border of the growth area.
(Figs. 1, 2, 5, living cultures. Figs. 3, 4, 7, Bouin fixed and Hem. eosine colored sections. Fig. 6 colored directly on the cover slip.)

could be seen near the explants until the 6th day after introduction of the virus.

In sections of tracheal fragments from infected cultures (5, 8 and 11 days after addition of virus) a continuous sheet of lining columnar epithelium was seen. In older cultures, it was often reduced to a monolayer of flattened but otherwise normal cells regularly dotted with cilia. On the other hand the underlying connective tissue and smooth muscle cells were heavily damaged, distinctly more than in the noninfected controls while the cells of the columnar epithelium, at different levels of the mucosa, retained a practically normal aspect.

4. *Cultures of monkey trachea and human bronchial tissues infected with adenovirus.* 10^4 ID_{50} (for HeLa cells) of the adenovirus added to the HeLa cell or monkey kidney cell cultures (whether trypsin dispersed or plasma imbedded) induced specific cell lesions within 48 hours and after 3 days nearly complete destruction of both kinds of cultures. A similar inoculation of the same amount of virus into 10-day-old cultures of monkey trachea did not appear to influence the ciliary beat which was still observed, on the periphery of the explants, 11 and in some cases 16 days after the infection. Relatively high virus titers were maintained throughout this period. This may be illustrated by the following figures (ID_{50}/ml of supernatant fluid in trachea epithelium cultures associated with HeLa cells): $10^{4.8}$ on the 6th day, $10^{4.2}$ on the 8th day, $10^{2.8}$ on the 11th day.

The epithelial sheets growing out from the tracheal fragments were attacked by the virus very slowly. The first specific cellular lesions (rounding of cells, intranuclear inclusions) began to appear at the periphery of the epithelial growth areas only 3 to 4 days after infection, and healthy cells could be seen near the fragments as late as 9 days after infection.

In sections of tracheal fragments after 26 days in culture and 16 days in presence of the adenovirus, normal lining epithelial ciliated cells were seen along with typically altered cells in the growth area.

A series of human bronchial tissue cultures were prepared from a patient without antibodies for the adenovirus type I and im-

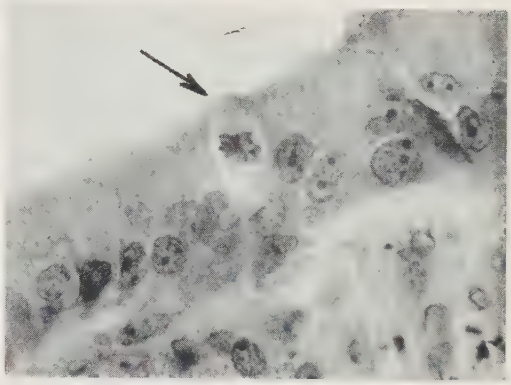

FIG. 7. 15 days' human bronchial culture; 5 days with adenovirus. Normal lining epithelium. One cell in mitosis (arrow).

planted additionally with HeLa cells. After inoculation of 10^4 ID_{50} per culture, all HeLa cells were destroyed, in 3 to 4 days the bronchial epithelial outgrowth also reacted to the virus but more slowly, whereas the epithelial sheets lining the fragments and inside the numerous glandular ducts, tubes or villi maintained a normal appearance during at least 6 days (Fig. 7).

Discussion. 1. The *in vitro* culture of the tracheal and bronchial lining tissue from different mammalian species was attempted by several authors(7,8,9,10). Improvements in tissue culture technics made it possible for us to maintain *in vitro* in an entirely heterologous medium adult human or simian respiratory tract tissues for a period of 30 days or more in a state of specific functional activity which was accompanied by a certain degree of proliferation.

2. The epithelial sheets extending from the explanted bronchial or tracheal fragments exceed considerably the surface of the original lining epithelium, mitotic figures are frequent and consequently these sheets may be considered as real growth areas. Thus, the ciliated cells appearing in these areas should be regarded as formed and differentiated *in vitro*. Nevertheless the general tendency of these growth areas which may be compared to cicatricial epithelium(11) is towards dedifferentiation and the ciliated cells definitely disappear from the growth zone between the 10th and the 14th days.

3. The reaction of the tracheal and bron-

chial epithelium to virus infection *in vitro* parallels this development. The periphery of the growth area, where the differentiated ciliated cells never appear, is the first to be attacked by the virus. On the other hand, the original lining epithelium which, more or less, retains its primary histological structure most efficiently resists the viral action. It must be emphasized that owing to the liquefaction of the plasma clot near the explants and also to the movement of the cilia the surfaces at the lining epithelium are in continuous contact with viral particles abundantly present in the fluid medium. Though the resistance to virus may be most easily demonstrated with the ciliated epithelium it appears to extend to other epithelial elements of the respiratory tract and particularly to the glandular epithelium at the different levels of the mucosa.

4. We have previously reported a similar relative resistance *in vitro* of the human adult tonsil epithelium(12) to poliovirus infection and proved that it was independent of the state of specific immunity of the tissue donor. This fact is confirmed by the present data obtained with the monkeys and at least one human case in which sera were free of specific viral antibodies. Moreover, in experiments with the monkey and the human embryo tissues the resistance of the differentiated respiratory epithelium to viruses could be contrasted with the sensitivity of the dedifferentiated cells originating from the same individual.

5. It may be mentioned that the same kind of resistance to virus action we are reporting here for human and monkey respiratory mucosa culture was observed in similar conditions in mouse trachea cultures and Mengo encephalomyelitis virus, an agent highly virulent and cytopathogenic for mouse tissue *in vitro*.‡ Thus the refractory state of the differentiated epithelium of the respiratory tract to cytopathogenic viruses *in vitro* may well represent a general phenomenon. It should also be noted here that Harford *et al.*(13,14) observed in living mice that the ciliated bronchial epithelium retained its full activity despite heavy and even lethal infection with a virulent influenza virus strain, though Francis and Stuart-Harris have shown in ferrets(15) as did Panthier *et al.* in mice(16) that this virus may cause necrosis in the respiratory mucosa cells.

6. The lining epithelium of the respiratory tract is considered by many not only as a way of entry for a number of viral infections but also for some of them as the site of their primary multiplication. However several attempts to use *in vitro* tissue cultures of the respiratory mucosa for the multiplications of respiratory viruses(17,18) gave rather unexpectedly negative results. These are easier to explain in the light of the reported data.

7. The *in vitro* resistance of the organized tissues of the respiratory tract to the cytopathogenic action of viruses obviously cannot exclude the possibility of latent infection or even virus multiplication and release from apparently normal cells(19). Closer investigations are here needed. Anyhow, our observations disclose that the well known general protective role of the ciliated epithelium ascribed to its mechanical and secretory functions may be extended to include a high degree of cellular resistance to virus action demonstrable even a long time after their explantation *in vitro*. Further work is necessary to understand the mechanism of this resistance and to determine whether it is or is not shared with other tissues which are able to maintain *in vitro* their functional differentiation.

Summary. 1. Fragments of human adult or embryonic bronchial mucosa and of monkey trachea cultivated *in vitro* in heterologous media maintain during several weeks their morphological and functional differentiation. 2. High concentrations of poliovirus or of adenovirus which rapidly destroyed the dedifferentiated growth of monkey kidney and HeLa strain cells in parallel or in mixed cultures had little or no destructive action on the differentiated respiratory epithelium and did not impair its ciliary activity.

‡ Unpublished data.

1. Kaplan, A. S., *Virol.*, 1955, v1, 377.

2. Scherer, N. F., Syverton, J. I., and Gey, G. O., *J. Exp. Med.*, 1953, v97, 695.

3. Barski, G., *Ann. Inst. Pasteur*, 1956, v90, 512.

4. Barski, G., Jezierski, A., and Lepine, P., *ibid.,* 1954, v86, 243.

5. Drouhet, V., and Costil, L., *ibid.,* 1956, v91, 818.

6. Barski, G., and Robineaux, R., *ibid.,* 1956, v90, 514.

7. Oppel, A., *Zentr. Zool. Allgem. Exp. Biol.,* 1913, v3, 209.

8. Carleton, H. M., *Philosoph. Trans. Roy. Soc. London,* series B 1925, v213, 365.

9. Strelin, Q. S., *Arch. exp. Zellfschg.,* 1929, v9, 297.

10. Schopper, W., *Wirchows. Arch.,* 1935, v295, 623.

11. Policard, A., *Précis d'Histologie physiologique,* 1950, p516.

12. Barski, G., Lepine, P., Monaci, V., and de Brion, G., *Ann. Inst. Pasteur,* 1953, v84, 825.

13. Harford, C. G., and Hamlin, A., *J. Exp. Med.,* 1952, v95, 173.

14. Harford, C. G., Hamlin, A., and Parker, E., *ibid.,* 1955, v101, 577.

15. Francis T., Jr., and Stuart-Harris, C. H., *ibid.,* 1938, v68, 789.

16. Panthier, R., Cataigne, G., and Hannoun, Cl., *Ann. Inst. Pasteur,* 1948, v75, 338.

17. Jordan, W. S., Jr., PROC. SOC. EXP. BIOL. AND MED., 1956, v92, 867.

18. Werner, G. H., *Méd. et Hygiène,* 1956, no. 346, 514.

19. Barski, G., *Science,* 1957, v125, 448.

Received August 8, 1957. P.S.E.B.M., 1957, v96.

Cuff for Use with Endpoint Devices for Estimation of Arterial Blood Pressure of the Rat.* (23486)

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Over the last several years a number of endpoint devices have been described, employing diverse principles, for estimation of the systolic or mean arterial blood pressure of the rat. Each of these has been designed to be used with an occlusive cuff by means of which the applied pressure may be varied. In our experience, most of the available cuffs have been unsatisfactory and have limited the confidence which one may place in data so obtained. After some experience with a commercially available inflatable cuff† designed to occlude circulation in the hind limb of the rat, it became apparent that, to avoid error introduced by the manner of placement, tightness of wrapping, etc., a cuff of fixed circumference is essential. Fig. 1 describes the preparation of such a cuff from the commercially available model, adhesive tape and a pair of snap fasteners.

In using the cuff, a few cautions should be observed. The width of the tape is critical; it should be just wide enough to reach from the foot to the thigh, thus accurately repositioning the inflatable portion of the cuff in subsequent measurements. Rats of different sizes require cuffs of proportionate size, *i.e.,* of different distances between the snap fasteners. A graded series, marked for identification, should be kept in stock and care taken that the same cuff is used for successive measurements with the same animal. When used on the tail, rather than on a limb, care should be taken that the cuff is replaced each time in the same position.

With these precautions, much of the subjective error of such measurements is reduced. Thus in a series of normal and renal hypertensive rats with systolic blood pressures ranging from 87-160 mm the following results were obtained: In 204 instances in which the blood pressure of a single animal was determined by more than one operator the mean deviation from the mean was within 6 mm, in 432 determinations in which a single operator performed repeated determinations on a single rat the mean deviation from the mean was

* This work was supported by grant from Life Insurance Medical Research Fund.

† Metro Industries, Long Island City, N. Y.

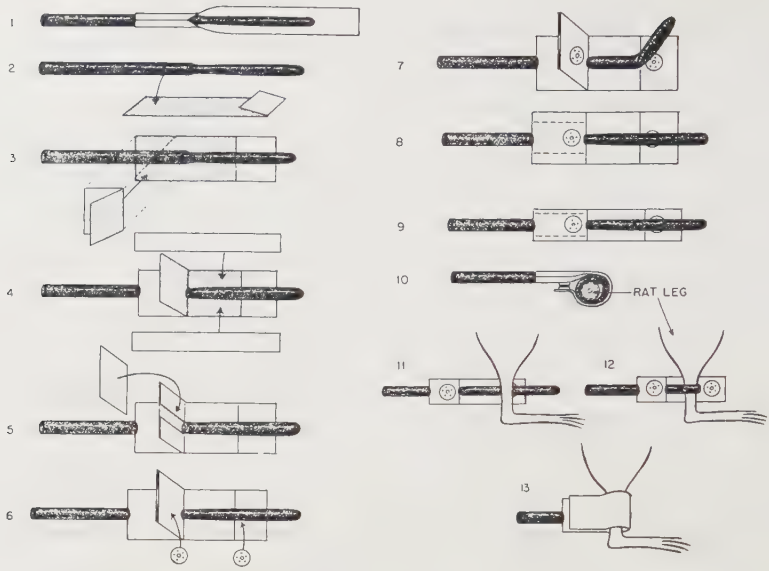

FIG. 1.

1. Commercial animal cuff for use with tensometer. Strip off the ribbon.
2. Place the bare rubber onto a piece of adhesive tape. Dots indicate sticky side of adhesive tape.
3. Cover part of non-inflatable end of the cuff with another strip of tape. For 150 g rats about 2.5 cm of inflatable cuff should be stuck to tape.
4. Cover exposed adhesive sections with 2 strips of tape.
5. Cover flap with a strip of adhesive tape.
6. Sew on a pair of small size snap fasteners. For 150 g rats, the snaps should be about 3.0 cm apart. 7-8. Fold down flap and sew. 9. Trim to size.
10. Top view of cuff in position.
11. Hold cuff so that free inflatable part is behind rat's leg.
12. Wrap free inflatable part of cuff around rat's ankle.
13. Snap the cuff shut, and rotate the entire cuff around rat's leg to insure even wrapping of inflatable portion.

within 3 mm.

Received August 12, 1957. P.S.E.B.M., 1957, v96.

Factors Influencing the Plate Method for Determining Abundance of Bacteria in Sea Water.*† (23487)

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Total number of bacteria in sea water as determined by plate counts is influenced by numerous factors(1). In this report some effects of medium composition and plating procedure are described.

Materials and methods. Sea water was collected at Long Branch, N. J., approximately 600 feet off shore in an area subject to neither pollution nor dilution. All samples were taken at high tide, packed in ice, and transported

* This investigation was supported in part by Research Grant from Natl. Inst. of Allergy and Infectious Diseases, N.I.H., Public Health Service.

† Journal Series Paper, Department of Agricultural Microbiology, N. J. Agric. Exper. Station, Rutgers, State University, New Brunswick, N. J.

TABLE I. Influence of Composition of Nutrient Medium on Plate Counts of Bacteria from Sea Water.

Medium constituents	Medium % composition* 1	2	3	4	5	6	7
Peptone	.5	.1	.5	.5	.5		.5
Gelatin						.3	
Dextrose		.1					
Glycerol					1.3		
Yeast extract			.01				
Beef extract				.3			
Ferric ammonium citrate				.01	.01	.01	
Na_2EDTA							.015
K_2HPO_4		.005		.01	.01	.01	.01
$FePO_4$	.01		.01				
$FeCl_3$							.002
Agar	1.5	1.5	1.5	1.5	1.5	1.5	1.5
Plate count as % of medium 1 †	100	6.2	123.5	36.6	47.4	20.5	38.9

* In 80% sea water. † Mean of 8 replicates.

to the laboratory for study. The water had a salinity of 2.9-3.0% and a reaction of pH 7.9-8.1. Dilution blanks were prepared with aged sea water, sterilized by autoclaving. Appropriate dilutions of the water samples were plated, using 8 replicate plates at each dilution. Counts were made after 7-10 days of incubation at 20°C.

Results. Table I shows the extent to which composition of the nutrient medium influenced the plate counts. The results are expressed as percentages of the average plate count obtained with medium 1. Medium 1 was described by ZoBell(2) and is used frequently in studies of the abundance and distribution of bacteria in marine environments. Medium 2 was recommended by Reuszer(3) for the cultivation of marine bacteria. The remaining five media were our own formulations. Medium 3 is medium 1 supplemented with yeast extract. Media 4-7 contained different organic substrates, but were similar in that phosphate was added as K_2HPO_4 and iron was present as the citrate or ethylenediaminetetraacetate chelate. All the media were approximately pH 7.5.

Maximum plate counts were obtained on medium 3. Medium 2 yielded the lowest counts. The increased count that resulted from the addition of yeast extract to ZoBell's medium confirms Jones' report(4) that yeast extract is "stimulatory" to marine bacteria.

Although medium 3 gave maximum plate counts, it contained a precipitate ($FePO_4$), the particles of which were easily confused with the smaller subsurface colonies. The enumeration of colonies on medium 3 was facilitated greatly when the liquified agar was left undisturbed until the precipitate settled, and only the clear supernatant was used for the preparation of plates. In media with chelating agents (media 4-7) difficulties due to precipitation were absent but plate counts were low. When medium 3 was prepared using distilled water, the plate counts were only 10% of those obtained when the medium was prepared with sea water.

The plating procedure also influenced the total number of colonies that developed from sea water on medium 3. Pour plates were prepared by adding agar to Petri dishes containing 1.0 ml of various dilutions of sea water. The medium and sea water were mixed thoroughly before the agar solidified. A second series of Petri dishes was prepared by adding approximately 15 ml of medium to each plate and allowing the agar to solidify. The surface of the agar was then inoculated with 0.1 ml of various dilutions of sea water, and the inoculum was distributed over the surface of the agar by rotating and tilting each plate. Petri dish tops made of metal and containing ab-

sorbent disks were used to desiccate the agar surface partially and thus to prevent spreading of colonies.

The number of bacterial colonies that developed on pour plates was consistently greater (30-40%) than the number that developed on surface-inoculated plates. This difference may have resulted from the growth of microaerophilic or anaerobic bacteria below the surface of the agar in pour plates. Colony counts were made more easily when a surface inoculum was employed; the colonies were larger, more uniform in size, and better distributed than those on pour plates.

Summary. The nutritional requirements of marine bacteria are so varied that no one nutrient medium can suffice for the growth of all. The influence of composition of the nutrient medium and plating procedure on the plate method for determining the number of bacteria in sea water was investigated. Seven media were tested. Maximum and reproducible plate counts were obtained with a medium containing 0.5% peptone, 0.01% yeast extract, 0.01% $FePO_4$, and 1.5% agar in 80% sea water. The number of colonies that developed on pour plates was 30-40% greater than the number that developed on surface inoculated plates.

The technical assistance of Miss E. Winkler is gratefully acknowledged.

1. ZoBell, C. E., *Marine Microbiology,* 1946, Chronica Botanica Co., Waltham, Mass.
2. ———, *J. Mar. Res.,* 1941, v4, 42.
3. Reuszer, H. W., *Biol. Bull.,* 1933, v65, 480.
4. Jones, G. E., *Bacteriol. Proc.,* 1957, Detroit, Mich., p16.

Received August 19, 1957. P.S.E.B.M., 1957, v96.

Attempts to Produce Antibodies to a Preparation of Polyglutamic Acid.* (23488)

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One of the recently proposed plasma expanders produced by synthetic methods has been poly-L-glutamic acid. Although many data have been obtained on the physico-chemical and physiological properties of synthetic polyamino acids(1), studies on the antigenicity of the polyamino acids themselves have been rather scant. The recent investigations of Stahmann and coworkers on the antigenicity in rabbits and guinea pigs of various polypeptides (polymers, copolymers and multico-polymers) either uncoupled or coupled to proteins (polypeptidyl proteins) are extremely significant and interesting(2,3). This report presents data concerning unsuccessful attempts to produce detectable antibodies in man, rabbits and guinea pigs with a preparation of poly-L-glutamic acid different from the one employed by Stahmann, *et al.*

Materials. The sodium poly-L-glutamate (PGA) (Lot No. S2035-186C) used in these studies was prepared by the American Cyanamid Company according to procedures of Dr. E. R. Blout and coworkers. The weight average molecular weight of the material as determined by viscosimetric and light scattering technics was approximately 80,000 which is equivalent to a degree of polymerization of 620. The preparation of polyglutamyl bovine plasma albumin (#693) PGA-BSA which was also used in testing the various sera, as described below, was kindly supplied by Dr. M. Stahmann. Analytical solutions of the above materials were prepared by dissolving the materials in pH 7.4 buffered 0.15 M NaCl and analyzing aliquots for N by the Markham modification of the micro Kjeldahl procedure(4).

* This investigation was supported by grant from Office of Surgeon General, Department of Army, upon recommendation of Subcommittee on Shock of Natl. Research Council. This is publication No. 136 of the Pathology Department, University of Pittsburgh School of Medicine.

TABLE I. Schedule of Injections of PGA into Human Volunteers.*

Group	No. of volunteers	Amt of PGA inj. per inj. (mg)	No. of inj.
1	6	2	5
2	6	5	5
3	6	15	5
4	6	50	5

* Saline solutions inj. intramusc.
No significant skin reactions were observed.

Methods. 1. *Immunization procedures*: a. *Man.* Healthy medical student volunteers were used. The procedures employed for obtaining pre- and post-immunization bleedings, and performing intracutaneous skin testing were those previously presented(5). The volunteers received intramuscular injections of varying concentrations of the materials over a period of 8 days as summarized in Table I. Post injection bleedings were obtained 3 weeks after the last injection. All serum samples were handled with sterile precautions and in addition "merthiolate" (0.01%) and phenol (0.25%) were added. b. *Rabbits.* Various immunization technics with alum precipitated PGA, or the PGA incorporated in complete adjuvant vehicle(6) were employed. For immunization with alum precipitated PGA (1.5 mg/ml) the schedule of 4 intravenous injections per week for 4 weeks as described in (7) was employed. The adjuvant preparation contained 25 mg PGA per ml. Rabbits received intramuscular injections of 1 ml of the material 3 times per week for 3 weeks. Cardiac bleedings were performed weekly for 8 weeks before reinjections of the PGA were resumed. The schedule of reinjections is presented in Table II. c. *Guinea pigs*: 1. Five guinea pigs (300-400 g) were given 1 ml injections (1 mg PGA/ml) intraperitoneally every other day for a total of 3 injections. Another group of 4 guinea pigs was given one intracardiac injection of 5 mg PGA and 3 other guinea pigs received one intracardiac injection of 10 mg PGA. Twenty-two days after the last injection all the guinea pigs were challenged with 10 mg PGA. The route of injection was either by cardiac puncture or injection into the foot or penile veins. 2. Six guinea pigs were given two 0.5 ml injections of the adjuvant mixture mentioned above. Bleedings were taken weekly for 8 weeks starting 3 weeks after the last injection. 2. *Testing of Antisera*: a. Agar Diffusion—All of the pre and post injection sera from rabbits and humans were analyzed both by the Oudin and Ouchterlony procedures of diffusion in agar(8). In the Oudin procedure the gellified media consisting of equal volumes of undiluted serum and 0.6% agar were overlayered with PGA (0.5 mg N/ml), PGA-BSA (0.5 mg N/ml), BSA (0.7 mg N/ml) or 0.15 M NaCl. The tubes were placed in a constant temperature room (20°C) and observed for 2 weeks. In the Ouchterlony procedure undiluted serum was placed in the center well and two-fold dilutions of PGA (2 mg/ml) were placed in the 6 surrounding wells. With PGA-BSA the concentrations employed ranged from 2.5 μg N to 124.0 μg N/ml. The diffusion plates were placed in the cold room and observed daily for 2 weeks. In addition to the above sera, a serum kindly supplied by Dr. M. Stahmann was also included in the experiments. b. Quantitative Precipitin Studies. 3.0 ml aliquots of the sera were set up with varying concentrations of polyglutamic acid (1.37 μg N to 12.5 μg N) and PGA-BSA (2.6-42.4 μg N). The mixtures were incubated at 37°C for ½ hour and placed in the cold for 2 weeks. The tubes were then centrifuged and analyzed for antibody by the quantitative precipitin method of Heidelberger and MacPherson(9). c. Passive Transfer Studies. 1. 1 and 2 ml aliquots of several pools of human and rabbit sera were injected intraperitoneally into guinea pigs. Forty-eight hours later they were injected intravenously with 5 mg PGA. 2. The passive cutaneous anaphylaxis reac-

TABLE II. Schedule of Injections of Rabbits with PGA.

		Methods of immunization		
		Course		
Group	Rabbits	I	II	III
I	6	Alum*	Alum	Adjuvant
II	6	Adjuvant†	Adjuvant	

All rabbits bled weekly for 8 wk before reinjections began.
* 1.5 mg PGA/ml. † 25 mg PGA/ml.

tion (PCA) as described by Ovary(10) was also attempted employing 12 guinea pigs (200-250 g). 0.1 ml volumes of the undiluted antiserum and 1→5 and 1→10 dilutions were injected intradermally in different sites. Four to 6 hours after the intradermal injection, 1.0 ml of a dye solution (0.25 ml 1% Evans Blue and 0.75 ml saline) was injected intravenously. Thirty minutes later a solution of PGA (1 mg) and Evans Blue was injected intravenously.

Results. Although positive reactions in agar were observed with the serum obtained from Dr. Stahmann and PGA-BSA, detectable antibodies were not observed in any of the sera obtained in this study by the technics of diffusion in agar, micro precipitin and passive anaphylaxis. In addition none of the guinea pigs "immunized" with PGA exhibited active anaphylaxis or cutaneous reactions when challenged with PGA.

Discussion. In view of what we know about the antigenicity of other materials such as pneumococcal polysaccharides(11), dextran(12), levan(13) and polyvinyl pyrrolidone(14), one is not surprised at the results obtained by Stahmann on the antigenicity of his preparation of PGA. However, the results presented in this report were quite unexpected. Of the possible factors contributing to the "non-antigenicity" of Blout's preparation of PGA the most plausible one appears to be the difference in the properties of the 2 polyglutamic acid preparations. The method of synthesis employed by Blout(15) results in a linear preparation with a molecular weight of 80,000, whereas Stahmann's preparation is branched and has a molecular weight of 13,000. It is not known how these differences in properties of the two preparations account for the differences in results. Further investigations with other polypeptides having different physico-chemical properties are necessary to answer this question.

Summary. Data have been presented to show that detectable antibodies were not observed in man, rabbit or the guinea pig against a preparation of polyglutamic acid. The differences in properties between Blout's and Stahmann's PGA have been mentioned as the possible reason for differences in antigenicity of the two preparations.

The author wishes to acknowledge the assistance of Dr. H. Mansmann with the skin testing and injections of the human volunteers. The author also wishes to thank Dr. Stahmann both for supplying the preparation of PGA-BSA and information about his observations on the antigenicity studies with Dr. Blout's preparation of PGA.

1. Blout, E. R., *et al.,* Reports to Subcommittee on Blood and Related Problems, Contract No. DA-49-007-MD-219.

2. Stahmann, M. A., Tsuyaki, H., Weiske, K., Lapresle, C., and Grabar, P., *Compt. Rend.,* 1955, v241, 1528.

3. Dellert, E., Buchanan, D. J., and Stahmann, M. A., *Fed. Proc.,* 1957, v16, 171.

4. Markham, R., *Biochem. J.,* 1946, v36, 790.

5. Maurer, P. H., PROC. SOC. EXP. BIOL. AND MED., 1953, v83, 879.

6. Ward, R., Rader, D., Lipton, M. M., and Freund, J., *ibid.,* 1950, v74, 536; Salk, J. E., and Youngner, J. S., *Fed. Proc.,* 1950, v9, 390.

7. Kabat, E. A., and Mayer, M. M., *Experimental Immunochemistry,* 1948, Charles C. Thomas, Springfield, Ill.

8. Oudin, J., *Methods in Medical Research,* 1952, v5, 335, Yearbook Publishers, Inc., Chicago, Ill.

9. Heidelberger, M., and MacPherson, C. F. C., *Science,* 1943, v97, 405; 1944, v98, 63.

10. Ovary, Z., and Biozzi, G., *Int. Arch. Allergy,* 1954, v5, 241.

11. Heidelberger, M., MacLeod, C. M., Kaiser, S. J., and Robinson, B., *J. Exp. Med.,* 1946, v83, 303; Heidelberger, M., and DiLapi, M. M., *J. Immunol.,* 1949, v61, 153; Heidelberger, M., DiLapi, M. M., Siegal, M., and Walter, A. W., *ibid.,* 1950, v65, 535; Heidelberger, M., MacLeod, C. M., DiLapi, M. M., *ibid.,* 1951, v66, 145; Heidelberger, M., Kendall, F. E., and Scherp, H. W., *J. Exp. Med.,* 1936, v64, 559.

12. Kabat, E. A., and Berg, D., *J. Immunol.,* 1953, v70, 514.

13. Allen, P. Z., and Kabat, E. A., *J. Exp. Med.,* 1957, v105, 383.

14. Maurer, P. H., *J. Immunol.,* 1956, v77, 105.

15. Blout, E. R., and Idelson, M., *J. Am. Chem. Soc.,* 1956, v78, 497.

Received August 19, 1957. P.S.E.B.M., 1957, v96.

Effect of Calcium Binding Substances on Rate and Duration of Narcosis. (23489)

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Marked changes in the intracellular content of calcium have been shown to accompany narcosis induced by fat solvent anesthetics (1,2,3). Although a cellular loss of calcium with liquefaction of the cortical gel forms the basis of the currently popular colloidal chemical theory of anesthesia(3), to our knowledge no data exist on the effect of calcium binding substances on the induction and duration of narcosis. This report is the result of preliminary studies directed toward this end.

Materials and methods. One hundred and twenty wild-type male guppies (*Lebistes reticulatus*) weighing between 65 and 85 mg were kept in an aquarium in Ringer's solution at 25°C and fed Long Life Tropical Fish Food once daily. The experimental groups consisted of 20 fish each. After an experiment the groups were returned to the aquarium for a minimum rest period of 24 hours before further experiments were undertaken. Various experiments were carried out with a 50 mg% solution of sodium pentobarbital in Ringer's solution (NaCl 6.7 g, KCl 0.14 g, $CaCl_2$ 0.12 g) at 25°C (pH 6.5-6.9) modified with respect to the calcium level and the presence or absence of disodium ethylenediamine tetraacetate (EDTA) or sodium citrate. The following experimental groups were used: Group A (control) Ringer's solution (1 mM Ca); Groups B, H, and I Ringer's citrate (1 mM Ca, 1.3 mM citrate); Group C balanced Ringer's citrate (1 mM Ca, 0.6 mM citrate); Group D Ringer's EDTA (1 mM Ca, 2 mM EDTA); Group E balanced Ringer's EDTA (1 mM Ca, 1 mM EDTA); Groups F and G calcium-free Ringer's; and Group J Ringer's with excess calcium (10 mM Ca). Groups B, H, and I contained an excess of citrate ion theoretically capable of binding calcium ion in the fish, while Group C contained a balanced calcium citrate solution. Group D contained an excess of EDTA theoretically capable of chelating 1 millimol of calcium ion, while Group E contained a balanced solution of calcium EDTA.

Groups G and J were pretreated for 4 hours prior to narcosis. Group G fish were pretreated in calcium-free Ringer's solution and Group J in Ringer's with excess calcium. The solution in which Group G fish were pretreated was quantitatively analyzed for calcium ion after pretreatment by the method of Clark and Collip(4). Group H fish were placed in Ringer's citrate for 1 hour prior to the induction of narcosis. Group I fish were placed in Ringer's solution with pentobarbital for 7.5 minutes then transferred to Ringer's citrate with pentobarbital. The fish were placed in the various solutions and the time required for induction of narcosis was recorded. The endpoint for narcosis was taken when the fish lost purposeful motion and were unable to right themselves. Following narcosis the fish were removed from the pentobarbital solution and placed in Ringer's solution, where the period of recovery was observed and recorded.

Results. The data for induction and recovery of pentobarbital narcosis in the presence of various calcium binding substances are summarized in Table I. The true mean induction times in minutes with the standard error for the various groups are: (A) 14.9 ± .64, (B) 7.8 ± .45, (C) 15.2 ± .54, (D) 7.1 ± .43, (E) 10.7 ± .45, (F) 13.7 ± .73, (G) 28.6 ± .82, (H) 28.7 ± .88, (I) 16.1 ± .93, and (J) 13.9 ± .86. A comparison of the mean induction times for Groups B and D with A (control) shows that excess sodium citrate (B) shortened the induction time of narcosis by 47.7%, while excess disodium EDTA (D) shortened it by 52.3%. Essentially no change was observed in Group C, a balanced solution of calcium citrate, in con-

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TABLE I. Summary of Data for Induction and Duration of Narcosis in the Presence of Calcium Binding Agents and in Calcium-Free Ringer's Solution.

Group	Sol.: Ringer's containing 50 mg % pentobarbital and modified as follows: Ca (mM)	Citrate (mM)	EDTA (mM)	Pretreat. time (hr)	Induction time (min.)	% difference compared to control (A)	Recovery time (hr)	% increase compared to control (A)
A	1				14.9 ± .64†		1.1 ± .08†	
B	1	1.3			7.8 ± .45	−47.7	1.4 ± .13	27.3
C	1	0.6			15.2 ± .54	+ 2.0	1.2 ± .12	9.1
D	1		2		7.1 ± .43	−52.3	3.4 ± .22	209.1
E	1		1		10.7 ± .45	−28.2	2.2 ± .22	100.0
F					13.7 ± .73	− 8.1	1.6 ± .22	45.5
G				4	28.6 ± .82	+91.9	1.2 ± .13	9.1
H	1	1.3		1	28.7 ± .88	+92.6	1.2 ± .09	9.1
I*	1	1.3			16.1 ± .93	+ 8.1	1.4 ± .16	27.3
J	10			4	13.9 ± .86	− 6.7	1.1 ± .10	0

* Pretreated in 50 mg % pentobarbital Ringer's solution for 7.5 min.
† S.E.

trast to the 28.2% shortening of induction time by a balanced solution of calcium EDTA (E). There was only a minimal decrease in induction time with calcium-free Ringer's solution (F), while pretreatment in calcium-free Ringer's for 4 hours prior to narcosis (G) lengthened the induction time by 91.9%. Similar results were observed by pretreatment with citrate for 1 hour (H) where the induction time showed a 92.6% increase. Quantitative analysis of the solution after the 4 hours treatment period of Group G fish showed that an average of .55 mg of calcium was released by each fish. Pretreatment with pentobarbital for one-half the mean normal induction time, *i.e.* 7.5 minutes (I), or with Ringer's solution containing a 10-fold increase in calcium for 4 hours (J) only slightly altered the induction time.

The true mean recovery times in hours with the standard error for the various groups are as follows: (A) 1.1 ± .08, (B) 1.4 ± .13, (C) 1.2 ± .12, (D) 3.4 ± .22, (E) 2.2 ± .22, (F) 1.6 ± .22, (G) 1.2 ± .13, (H) 1.2 ± .09, (I) 1.4 ± .16, and (J) 1.1 ± .10. Marked prolongation of the mean recovery time was observed with Ringer's EDTA (D) which showed an increase of 209.1%, with balanced EDTA (E) which showed an increase of 100%, and with calcium-free Ringer's solution which showed a 45.5% increase.

Discussion. The presence of calcium binding agents(5,6) during pentobarbital narcosis shortened the period of induction by approximately 50%. While these results are generally in accordance with the concept that changes in calcium metabolism are of importance in narcosis(3), there are several findings which are of considerable interest.

Procedures capable of lowering the calcium level of the fish prior to narcosis by either pretreatment in Ringer's containing 1.3 millimols of citrate or in calcium-free Ringer's solution resulted in a prolongation of approximately 90% in the induction time. On the other hand, pretreatment of the fish for one-half the mean induction time of the control group in Ringer's solution with pentobarbital, prior to being transferred to Ringer's citrate with pentobarbital, resulted in essentially no change in induction time. Thus it appears that the synergistic effect of hypocalcemia on pentobarbital narcosis depends on the simultaneous introduction of both hypocalcemia and pentobarbital. The results observed with calcium-free Ringer's and Ringer's citrate both with and without pretreatment suggest that the rate of induction of hypocalcemia by diffusion or chemical binding may be of prime importance in altering the induction time of narcosis.

The failure of balanced calcium citrate solution to shorten the induction time and prolong recovery time to any extent in contrast to the shorter induction time and longer recovery time with balanced EDTA corroborates the observation of Bersin, *et al.*(7) that EDTA exerts a synergistic effect on barbiturate nar-

cosis. It is interesting to note that the excess EDTA solution, which was capable of chelating calcium, had a more pronounced effect on shortening induction time and prolonging recovery time than the balanced EDTA solution. The binding of calcium ions during the induction of narcosis by either citrate or EDTA resulted in a prolongation of recovery time. Thus, it appears that ionized calcium is of importance in the rate of recovery from narcosis. The anomalous results with reference to the recovery times observed when narcosis was induced in calcium-free Ringer's solution and following a 4 hour pretreatment period in this solution remain unexplained at the present time.

Pretreatment of the fish in calcium-free Ringer's solution resulted in a loss of calcium ions as shown by quantitative analysis. This is in accord with observations of Krogh(8) who showed that various ions may be removed by diffusion, primarily across the gill capillaries, when fish are placed in ion-poor solutions. The inability of Ringer's solution containing excess calcium to modify the induction of narcosis even after the 4 hour pretreatment period may indicate either that excess intracellular calcium has no effect on narcosis or that calcium diffuses across cellular membranes and becomes incorporated into the cell with great difficulty.

Summary. 1. The induction time of pentobarbital narcosis was shortened by 50% when carried out in the presence of calcium binding substances. 2. Removal of calcium by binding with citrate ion or pretreatment of the fish in calcium-free Ringer's solution prior to induction of pentobarbital narcosis resulted in a prolongation of the induction time (90%). 3. Balanced calcium EDTA solution shortened the induction time (30%) and lengthened the recovery time (100%) in spite of its theoretical inability to chelate calcium, while excess EDTA caused a greater shortening in induction time (50%) and lengthening in the recovery time (200%). 4. Excess calcium exerted no effect on induction or recovery of pentobarbital narcosis.

1. Mazia, D., and Clark, J. M., *Biol. Bull.,* 1936, v71, 306.

2. Berwick, M. C., *J. Cell. and Comp. Physiol.,* 1951, v38, 95.

3. Heilbrunn, L. V., *An Outline of General Physiology, 3rd ed.,* Philadelphia, Saunders Co., 1952.

4. Clark, E. P., and Collip, J. B., *J. Biol. Chem.,* 1925, v63, 461.

5. Shelling, D. H., and Maslow, H. L., *ibid.,* 1928, v78, 661.

6. Technical Bulletin, *The Versenes,* Bersworth Chemical Co., Framingham, Mass., 1954.

7. Bersin, T., Mueller, A., and Schwartz, A., *Arzneimittel-Forsch.,* 1954, v4, 119.

8. Krogh, A., *Osmotic Regulation of Aquatic Animals,* Cambridge University Press, 1939, 130.

Received August 26, 1957. P.S.E.B.M., 1957, v96.

Nutritional Requirements of *Micrococcus freudenreichii.** (23490)

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As a preliminary step in the investigation of certain enzyme systems in *Micrococcus freudenreichii,* it became desirable to grow the organism in a chemically defined medium which was capable of producing a high yield of cells. When a search of the literature failed to reveal the necessary information concerning the nutritional requirements of this organism, an investigation of these requirements was undertaken.

Materials and methods. Cultures and inocula. The culture employed in the studies was *Micrococcus freudenreichii* ATCC 8459. It was maintained on a stab in stock medium containing peptonized milk, 0.1%; tryptone, 0.1%; filtered tomato juice, 20%; and agar,

* This study was supported by grant-in-aid from Robert A. Welch Fn.

TABLE I. Composition of Basal Medium.

Component	Amt/100 ml of double strength medium
Sodium acetate • $3H_2O$	2.4 g
Ammonium chloride	.3
Sucrose	3.0
Adenine sulfate	3 mg
Guanine • HCl	3
Xanthine	3
K_2HPO_4	100
KH_2PO_4	100
$MgSO_4 \cdot 7H_2O$	40
NaCl	2
$FeSO_4 \cdot 7H_2O$	2
$MnSO_4 \cdot H_2O$	1.5
DL-Alanine	80
L-Arginine • HCl	48.5
L-Asparagine*	80
DL-Aspartic acid	20
L-Cysteine • HCl	10
DL-Glutamic acid	60
Glycine	20
L-Histidine • HCl	12.5
L-Isoleucine	50
DL-Leucine	50
L-Lysine • HCl	50
DL-Methionine	20
DL-Phenylalanine	20
L-Proline	20
DL-Serine	10
DL-Threonine	40
DL-Tryptophan	8
L-Tyrosine	20
DL-Valine	50
Calcium pantothenate	.5
Thiamine • HCl	.5
Riboflavin	.5
Nicotinic acid	1
Pyridoxine • HCl	1
p-Aminobenzoic acid	.1
Biotin	1 μg
Folic acid	10

* *L*-Asparagine was included in basal medium only for those experiments which tested amino acid requirements of the organism. When it was found that slightly better growth was obtained in the absence of asparagine, this compound was omitted from subsequent experiments.

1.5%. Transfers were made at monthly intervals and were incubated at 26°C for approximately 24 hours. Inoculum medium was identical to the above with agar omitted. A transfer was made from stock culture approximately 8 hours before the inoculum was needed, and this was incubated at 26°C. The cells were centrifuged, washed twice in sterile 0.9% saline solution, then resuspended in the saline solution to give a reading of 70 to 80% transmittance in the Bausch and Lomb photoelectric colorimeter with a 660 mμ filter. Each assay tube was inoculated with a single drop of this suspension. *Basal medium.* Composition of the basal medium is shown in Table I. Final pH of the medium was adjusted to 7.0. *Procedure for growth tests.* In determining essentiality of various nutrients for growth, each compound was omitted individually from the basal medium. Six ml of single strength basal medium were added to each 18 x 150 mm culture tube, covered with aluminum caps, and autoclaved five minutes at 121°C. After the tubes had cooled, they were inoculated and incubated at 26°C until maximum growth. Growth was determined by turbidimetric reading in a Bausch and Lomb photoelectric colorimeter using a 660 mμ filter.

Results. Effect of CO_2. In early experiments, it was found that growth was often slow and light, and that results were difficult to duplicate. When CO_2 was supplied by the addition of a filter sterilized solution of $NaHCO_3$, growth was found to be much more rapid, heavier, and more reproducible from one experiment to another. In view of these effects, 0.2 mM of $NaHCO_3$ was routinely included in each 6 ml culture by addition of a filter sterilized solution to the autoclaved medium.

The stimulatory effect of CO_2 on the growth of *M. freudenreichii* is similar to that observed by previous workers with various other microorganisms(1,2) and to results obtained in previous work in our laboratory with *Streptococcus bovis*(3,4). In the latter instance, however, CO_2 was implicated in the synthesis of amino acids, both by the finding that $C^{14}O_2$ was rapidly incorporated into the carbon skeleton of amino acids, and by the fact that CO_2 was not required when the medium contained a complete mixture of amino acids. It is unlikely that CO_2 functions in the synthesis of amino acids in *M. freudenreichii* when a complete mixture is supplied. The likelihood exists that CO_2 functions in other synthetic pathways, however.

Amino acid requirements. In repeated experiments, the omission of any one of the following amino acids resulted in growth failures: arginine, glutamic acid, histidine, leucine, methionine, threonine, tryptophan, and

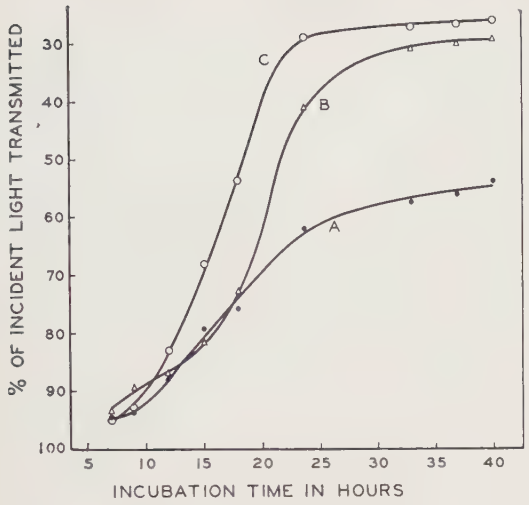

FIG. 1. Growth rates of *M. freudenreichii* on 3 complete amino acid mixtures. A, mixture in basal medium. B, mixture of Henderson and Snell(6). C, mixture shown in Table II.

valine. If, however, $NaHCO_3$ were omitted from the medium, the organism required aspartic acid in addition to the amino acids listed above. Asparagine was incapable of replacing aspartic acid in the absence of CO_2. It is well known that commercial samples of leucine, isoleucine, and valine are sometimes cross-contaminated with each other. The leucine, isoleucine, and valine used in these studies were free of such contamination as shown by assay with *Lactobacillus plantarum* (*Lactobacillus arabinosus* 17-5).

Attempts were made to grow *M. freudenreichii* in a medium identical to the basal medium except that the complete amino acid mixture was replaced by a mixture containing only the essential amino acids in the same ratio in which they occur in the complete mixture. These efforts were unsuccessful, but moderate to heavy growth could be obtained from the essential amino acids after prolonged incubation by altering the ratios of histidine to arginine and of leucine to valine to values of 3:1 or 4:1. The addition of alanine, glycine, isoleucine, phenylalanine, and serine to the 8 essential amino acids produced more rapid growth, but the lag phase was still longer than when the complete amino acid mixture was supplied.

Because one of the objectives of the investigation was to find a medium which would support vigorous growth of *M. freudenreichii*, different complete mixtures of amino acids were tested for ability to produce heavy cell yields. Fig. 1 shows the results obtained when different amino acid mixtures were compared in the presence of $NaHCO_3$. Mixture A was contained in the basal medium and is similar to that of Sauberlich and Baumann (5). Mixture B is that of Henderson and Snell(6), and Mixture C has the composition shown in Table II. All 3 mixtures are very similar qualitatively, but show considerable variations in the ratios of individual amino acids. It is obvious that growth of *M. freudenreichii* is affected to a considerable extent by the relative amounts of various amino acids in the medium.

Vitamin requirements. When all vitamins were omitted, little if any growth occurred. Upon the individual omission of each vitamin, repeated tests showed that absence of no single vitamin caused complete growth failures. The absence of pantothenic acid, folic acid, and riboflavin reduced the growth drastically, however, and niacin and pyridoxine both appeared to possess a slight stimulatory effect, although not nearly so marked as that of the first 3 compounds. Thiamine, biotin, and *p*-amino-benzoic acid could be left out without any effect on growth.

TABLE II. Composition of Amino Acid Mixture C.

Component	mg/100 ml of double strength medium
DL-Alanine	68.7
L-Arginine · HCl	83.1
L-Aspartic acid	34.4
L-Cysteine · HCl	17.2
L-Glutamic acid	103.0
Glycine	34.4
L-Histidine · HCl	21.3
DL-Isoleucine	85.9
DL-Leucine	85.9
L-Lysine · HCl	85.9
DL-Methionine	34.4
DL-Phenylalanine	34.4
L-Proline	34.4
DL-Serine	17.2
DL-Threonine	68.7
DL-Tryptophan	13.7
L-Tyrosine	34.4
DL-Valine	85.9

The possibility existed that the 3 most stimulatory vitamins were, in reality, essential to growth of the organism, and that small amounts were being supplied as contaminants from other constituents of the medium. In order to test the possibility that the vitamins used were cross-contaminated, a complete lot of new vitamins was opened and the tests were repeated several times, with the same results as above. If contamination were involved, it seems likely that it came from some constituent of the medium besides the other vitamins. Alternatively, the organism may be capable of synthesizing pantothenic acid, folic acid, and riboflavin at sub-optimal rates which permit limited growth.

Purine and pyrimidine requirements. The omission of all purines and pyrimidines from the otherwise complete medium resulted in growth failures. It was found that individual addition of adenine, guanine, xanthine, hypoxanthine, adenosine, guanosine, or xanthosine supported heavy growth of *M. freudenreichii*. Thymine, uracil, cytosine or thymidine were incapable of supporting growth. It appears, then, that the requirement of the organism can be met by any purine or its riboside, but not by pyrimidines or their pentosides.

Carbohydrate requirements. Out of 21 carbohydrates tested, heavy growth was supported by glucose, maltose, trehalose, fructose, mannose, sucrose, lactose, starch, and galactose. It thus appears that the organism can use a wide variety of carbohydrates as its energy source.

Summary. The nutritional requirements of *Micrococcus freudenreichii* 8459 for amino acids, vitamins, purines, pyrimidines, and carbohydrates have been studied. Three complete mixtures of amino acids gave considerable variation in growth, indicating that the organism is sensitive to the ratios of the various amino acids present. Eight amino acids were indispensable for growth. No single vitamin was shown to be essential, but growth was light in the absence of pantothenic acid, riboflavin, or folic acid. The single addition of any of the purines or purine ribosides tested supported heavy growth, but none of the pyrimidines or their pentose derivatives tested permitted growth in the absence of a purine. Of 21 carbohydrates tested, 9 were capable of serving as an energy source for *M. freudenreichii*. Growth was greatly improved when CO_2 was supplied to the medium as $NaHCO_3$.

1. Lyman, C. M., Moseley, O., Wood, S., Butler, B., and Hale, F., *J. Biol. Chem.*, 1947, v167, 177.

2. Huhtanen, C. N., Carleton, F. J., and Roberts, H. R., *J. Bact.*, 1954, v68, 749.

3. Prescott, J. M., and Stutts, A. L., *ibid.*, 1955, v70, 285.

4. Prescott, J. M., Ragland, R. S., and Stutts, A. L., *ibid.*, 1957, v73, 133.

5. Sauberlich, H. E., and Baumann, C. A., *J. Biol. Chem.*, 1948, v176, 165.

6. Henderson, L. M., and Snell, E. E., *ibid.*, 1948, v176, 15.

Received August 26, 1957. P.S.E.B.M., 1957, v96.

Effects of High Fat and Carbohydrate Diets on Rat Liver Phosphatase.* (23491)

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The fact that adaptation of rats to a high fat diet increases the rate of utilization of fat and decreases that of carbohydrate during a subsequent fasting period(1,2) suggested the possibility that with the change in metabolic pattern, the activity of some enzymes involved in carbohydrate metabolism may also be altered. We considered particularly alkaline phosphatase because several authors had shown that the phosphatase activity of some tissues underwent changes due to starvation,

* Supported by grant from Dairy Industry Advisory Board of State of California.

protein depletion, and the feeding of different diets(3,4,5,6). Bellini and Cera(7) reported an increase of phosphatase activity in the rat intestine after the ingestion of neutral fats. This effect varied inversely with the chain length for butyric, lauric and stearic acids. Oleic acid was more active in increasing the activity than were the saturated fatty acids (8). It has also been shown that alkaline phosphatase activity in rat intestine decreases during fasting and increases during feeding. Furthermore, a meal containing fat causes a greater increase in lymph and plasma alkaline phosphatase activity than does a fat-free meal (9). The increase of alkaline phosphatase in the intestinal lymph following a fat containing meal does not occur in rats with biliary fistula or after ligation of the bile duct(10). Histochemical determinations of the acid and alkaline phosphatase activities of the small intestine of the rat have failed, however, to show any difference in activity after feeding high carbohydrate, high fat or high protein diets(11). In human subjects and in albino rats, serum alkaline phosphatase was lowered by fasting and raised by ingestion of fat. Proteins and carbohydrate had no effect(12).

In the following experiments liver phosphatase activity was studied during the absorptive and post-absorptive periods in rats adapted to a high fat diet, and in non-adapted rats fed a single high fat meal.

Materials and methods. Male adult Wistar rats were used throughout these experiments. They were housed in individual cages and fed *ad lib.* All diets were compounded so that casein supplied 15.5% of the total caloric content. These diets maintained satisfactory growth in all cases.

Composition of high carbohydrate diet: Casein 315 g, USP salt mixture #5 105 g, cane sugar 105 g, corn starch 1575 g, choline HCl 3 g, methyl linoleate 3 g, corn oil 15 g.

Composition of the high fat diet: Casein 315 g, USP salt mixture #5 96 g, cane sugar 60 g, butter fat 684 g, ruffex 60 g, choline HCl 3 g, methyl linoleate 3 g. corn oil 15 g. All diets were supplemented with vitamins to previously established levels (13). For determination of alkaline phosphatase activity, rats were sacrificed by means of a blow on the neck. The median lobe of the liver was removed, weighed, and homogenized in a Potter-Elvehjem homogenizer at high speed for 3 minutes. A 20% homogenate was made by diluting with ice cold distilled water. During the homogenizing period the tube was immersed in ice cold water. The homogenate was then filtered through a thin layer of surgical cotton in the cold room. The phosphatase activity was measured immediately. The Bessey, Lowry and Brock method(14) which uses p-nitrophenol phosphate† as a substrate was modified in order to avoid the turbidity interference due to the presence of insoluble particles. We therefore adapted the method for determining phosphatase activity in tissue homogenates as follows: For measuring alkaline phosphatase activity 1 ml of the glycine buffered substrate previously adjusted to pH 9.4 was pipeted into a test tube. It was kept for 5 minutes in a water bath at 38°C. An aliquot of liver homogenate (0.1-0.2 ml) was pipeted into the tube and the solution incubated at 38°C for thirty minutes. At the end of the incubation period the enzyme was inactivated by addition of 2 ml of a 10% solution of trichloroacetic acid. Sufficient water was then added to bring the final volume to 10.9 ml. The sample was filtered through Whatman #40 paper and 0.2 ml of 40% NaOH was then added to the filtrate. The color developed was read at a wavelength of 420 mμ with a Lumetron colorimeter. Acid phosphatase activity was measured by the same procedure at pH 4.8 using a citric acid buffer. This method, which eliminates the correction for turbidity, gave reproducible results throughout all the experiments. The activity was expressed in Bessey-Lowry units. A "B-L" unit or millimolar unit is the amount of phosphatase which will liberate 1 mM of p-nitrophenol per hour/1000 g of liver.

Results. Table I shows the effects of a 33-day adaptation to high fat and high carbohydrate diets. A group fed Rockland rat pellets, which is essentially a high carbohydrate diet (4.0% fat), is also included. The rats

† Supplied by Sigma Chemical Co., St. Louis, Mo.

TABLE I. Effect of Prolonged Feeding of a High Fat and High Carbohydrate Diet on Liver Phosphatase Activity during Absorption.

Group	No. rats	Diet	Adaptation period	B-L units alkaline phosphatase	B-L units acid phosphatase
I	5	Rockland rat pellets		14.0 ± .3	
II	7	High CHO	33 days	14.6 ± .9	498 ± 20
III	7	" fat	33 "	20.2 ± 2.7	517 ± 12

were sacrificed during the absorptive period, at which time food was still present in their stomachs and intestines.

Alkaline phosphatase activity was significantly increased for the high fat group, as compared to the other two. No difference was evident between the high carbohydrate and the Rockland fed animals.

No significant changes were noticed in the acid phosphatase activity for the high carbohydrate and high fat groups.

The effect of a 12-hour fast in animals which were previously fed a high fat and high carbohydrate diet for 30 days was investigated in a second group of experiments (Table II). No difference was observed in the liver alkaline phosphatase activity.

Finally Table III shows the phosphatase activity after a single meal of the high fat diet was consumed by animals previously fed the Rockland stock diet. The rats in this experiment were sacrificed during the absorptive period. As in experiment 1 the alkaline phosphatase activity was increased in comparison to the activity in controls fed the Rockland diet.

Discussion. The feeding of a high fat diet increased the alkaline phosphatase activity of rat liver during the absorption period. Acid phosphatase activity did not show any significant change. This increase of activity occurred only during absorption. Adaptation to high fat diets failed to produce any increase which would persist into the post-absorptive period. Thus it would appear that during fat absorption a transient increase of alkaline phosphatase activity occurs in the liver. The feeding of a single high fat meal increased the alkaline phosphatase activity of the liver during absorption in the same manner as did feeding a high fat diet over a considerable period of time.

These findings are in accord with those of other authors, who demonstrated that alkaline phosphatase activity of intestine and lymph increases during the feeding of fats.

TABLE II. Effect of Prolonged Feeding of High Fat and High Carbohydrate Diet on Liver Phosphatase Activity after 12 Hr Fasting.

Group	No. rats	Diet	Adaptation period	B-L units alkaline phosphatase
I	7	High CHO	30 days	14.5 ± .8
II	6	" fat	30 "	14.5 ± 3.4

TABLE III. Effect of a Single Meal of High Fat Diet on the Liver Alkaline Phosphatase Activity during Absorption.

Group	No. rats	Diet	B-L units alkaline phosphatase
I	8	High fat	23.3 ± 3.5
II	5	Rockland rat pellets	14.0 ± .3

Summary. 1. Feeding of a high fat diet increased the alkaline phosphatase activity of rat liver during the absorptive period. This activity returned to normal after a 12-hour fast. Adaptation to a high fat diet for 30 days did not alter this pattern. 2. These results indicate that the increase of alkaline phosphatase activity after feeding of fat is a transitory result of fat absorption and not a consequence of change of metabolic pattern due to fat adaptation. 3. The method using p-nitrophenol phosphate as a substrate was modified for determining phosphatase activity in tissue homogenates.

The author wishes to thank Dr. Ernest Geiger for his suggestions and counsel.

1. Samuels, L. T., Reinecke, R. M., and Ball, H. A., *Endocrinol.*, 1942, v31, 42.

2. El Rawi, I., Dissertation, Univ. of So. California, 1953.

3. Ross, M. H., and Batt, W. G., *J. Nutrition,*

1956, v60, 137.

4. Rosenthal, O., Fahl, J., and Vars, H., *J. Biol. Chem.,* 1952, v194, 299.

5. Ely, J. O., and Ross, M. H., *Nature,* 1951, v168, 323.

6. Scrinwasan, P. R., and Patwardham, U. N., *Indian J. Med. Research,* 1955, v43, 1.

7. Bellini, L., and Cera, B., *Pathologica,* 1940, v32, 195. (See *Chem. Abst.,* v34, 7350)

8. Tuba, J., and Dickie, N., *Can. J. Bioch. and Physiol.,* 1954, v32, 621.

9. Flock, E. V., and Bollman, J. L., *J. Biol. Chem.,* 1948, v175, 439.

10. ———, *ibid.,* 1950, v184, 523.

11. Stenram, U., *Acta Anat.,* 1954, v20, 101.

12. Sukumaran, M., and Bloom, W. L., Proc. Soc. Exp. Biol. and Med., 1953, v84, 631.

13. Geiger, E., and El Rawi, I., *Metabolism,* 1952, v1, 145.

14. Bessey, O. A., Lowry, O. H., and Brock, M. J., *J. Biol. Chem.,* 1946, v164, 321.

Received August 30, 1957. P.S.E.B.M., 1957, v96.

Androgen and the *myo*-Inositol Content of Male Accessory Organs of the Rat.* (23492)

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The function of inositol in animal and plant physiology has been the subject of many investigations since its discovery more than one hundred years ago by Scherer(1). Inositol is required for growth of certain yeasts(2) and mice(3), and a dietary deficiency of this factor in hamsters is associated with reproductive disturbances(4). The importance of inositol as a lipotropic substance has been established(5). Furthermore, it has been demonstrated that *myo*-inositol is an essential growth factor for normal and malignant human cells in tissue culture(6,7). Mann(8) has reported the occurrence of relatively large quantities of free inositol in the seminal vesicles of the boar. Inositol was present to the extent of 2 to 3 g per 100 ml of seminal vesicle secretion.

The investigation presented here deals with the *myo*-inositol content of the male accessory organs of the rat during growth and the influence of androgen on the *myo*-inositol content of these organs. The significance of inositol in male accessory organs and secretions is obscure. It may be involved in the carbohydrate metabolism of these organs. Stetten and Stetten(9) demonstrated that deutero-labeled inositol is converted to glucose by the rat. Daughaday *et al.*(10) using glucose uniformly labeled with C^{14} showed that in the immature rat and chick embryo endogenous synthesis of inositol occurs.

Methods. Male rats of the Holtzman strain were used in this investigation and were fed Purina Laboratory Chow and raw carrots. The seminal vesicles, coagulating glands, dorsal and ventral prostates, and Cowper's glands were used in this study. Secretion-free organs were prepared by splitting glands lengthwise and removing the secretion by blotting on filter paper. Bilateral castration was performed on animals in which the effects of gonadectomy and androgen replacement were studied. A period of at least 20 days was allowed following castration for the regression of the male accessory organs. Testosterone propionate in oil† was given subcutaneously in daily doses of 500 μg. The selective uptake of *myo*-inositol by the seminal vesicles and dorsal prostates of castrate rats was studied. *Myo*-inositol was administered by stomach tube (25 mg per 100 g body wt) to fasting castrate and hormone-treated castrates 12, 8 and 4 hours before they were sacrificed. *Myo*-inositol was isolated and

* Journal Paper No. J-3256 of the Iowa Agric. and Home Economics Exp. Station, Ames, Ia. Project No. 1325. Acknowledgement is made of research grants, Office of Naval Research and Nat. Cancer Inst. of U.S.P.H.S., Department of Health, Education and Welfare.

† Peranderen, Ciba Pharmaceutical Products, Summit, N. J.

TABLE I. Effect of Acid Hydrolysis on Recovery of *Myo*-inositol Added to Rat Accessory Organs.

Organ	Sample	μg *myo*-inositol Added	Sample	Recovered	% recovery	Avg % recovery
Seminal vesicles	1	0	9161			
	2	1400	10492	1331	95	
Coagulating glands	1	0	1400			
	2	2800	4140	2740	98	
Dorsal prostates	1	0	3450			
	2	1000	4550	1100	110	101

identified from pooled accessory organs of mature rats using the method of Halliday and Anderson(11). The isolated compound gave a melting point of 222-223°C. Furthermore, *myo*-inositol hexacetate (mp 217°C) was prepared from the isolated *myo*-inositol. Microbiological assays for inositol were carried out using *Neurospora crassa* 37401 according to a procedure described by Beadle(12). Accessory organs to be assayed were removed from the animals, weighed and hydrolyzed in 4N sulphuric acid in an autoclave for 2 hours at 15 lb pressure. Hydrolyzates were adjusted to a pH of 5.5 with sodium hydroxide and diluted to volume prior to assay. Recovery assays were carried out in which different levels of *myo*-inositol were added to samples of seminal vesicles, coagulating glands, and dorsal prostates before hydrolysis. These data are presented in Table I. It is noted that the average recovery was 101%. These data would suggest that little, if any, *myo*-inositol is lost during the hydrolysis procedure. During this investigation it was found that the *myo*-inositol present in the male accessory organs of the rat is available to *Neurospora crassa* without prior acid hydrolysis. Tissue may be prepared for assay by homogenization and subsequent centrifugation or filtration or dialysis.

Results. Animals were selected at different stages of maturity to furnish data in the *myo*-inositol content of rat male accessory organs during development. These data are presented in Table II. The effects of castration and androgen replacement on the *myo*-inositol content of rat accessory organs are presented in Tables III and IV, respectively. Data are presented in Table V showing the influence of androgen replacement and stomach-feeding of *myo*-inositol on the seminal vesicles and dorsal prostates of the castrate rat.

Discussion. The data presented in Table II show increases in weights and *myo*-inositol contents of male accessory organs during growth of the rat. The marked differences noted between Groups 4 and 5 occurred at the time of increased gonadal androgen production at puberty. The *myo*-inositol concentration increased during growth of the seminal vesicles, coagulating glands and dorsal prostates whereas a decrease in concentration was noted in the dorsal prostates and Cowper's

TABLE II. Changes in *Myo*-inositol Content of Male Accessory Organs during Growth.

		Seminal vesicles		Coagulating glands		Dorsal prostates		Ventral prostates		Cowper's glands	
Animals per group	Body wt (g)	Wt (mg)	Inositol (μg)	Wt (mg)	Inositol (μg)	Wt (mg)	Inositol (μg)	Wt (mg)	Inositol (μg)	Wt (mg)	Inositol (μg)
10	63*	8*	20*	3*	10*	13*	58*	29*	75*	1*	2*
10	88	12	26	5	14	18	81	42	97	2	3
10	122	22	59	7	29	26	136	46	135	3	5
10	147	27	69	10	49	32	162	59	162	5	7
5	190	90	269	23	87	57	332	119	261	19	21
5	219	271	763	55	203	101	576	174	366	33	31
5	262	466	1613	72	275	112	672	225	463	42	42
5	385	945	5239	151	664	244	1704	594	978	67	44

* Mean.

TABLE III. Effect of Castration on Weight and *Myo*-inositol Content of Secretion-Free Male Accessory Organs of the Rat.

Days after castration	Animals per group	Seminal vesicles		Coagulating glands		Dorsal prostates		Ventral prostates		Cowper's glands	
		Wt (mg)	Inositol (μg)	Wt (mg)	Inositol (μg)	Wt (mg)	Inositol (μg)	Wt (mg)	Inositol (μg)	Wt (mg)	Inositol (μg)
0	7*	218 ± 7†	520 ± 41	65 ± 3	226 ± 28	108 ± 10	790 ± 64	181 ± 14	327 ± 61	29 ± 3	40 ± 9
2	5	158 ± 11	426 ± 68	49 ± 2	225 ± 21	92 ± 6	564 ± 30	118 ± 17	326 ± 56	22 ± 1	27 ± 5
10	10	116 ± 10	221 ± 13	31 ± 3	90 ± 11	55 ± 5	233 ± 31	40 ± 4	98 ± 14	25 ± 1	12 ± 1
20	5	62 ± 1	145 ± 8	17 ± 1	41 ± 2	25 ± 2	90 ± 13	27 ± 2	61 ± 5	8 ± 1	

* Mean wt animals 319 ± 5 g. † Mean ± S.E.

TABLE IV. Effect of Testosterone Propionate on Weight and *Myo*-inositol Content of Accessory Organs of Castrate Rats.

Days of hormone inj., 500 μg daily	Animals per group	Seminal vesicles		Coagulating glands		Dorsal prostates		Ventral prostates		Cowper's glands	
		Wt (mg)	Inositol (μg)	Wt (mg)	Inositol (μg)	Wt (mg)	Inositol (μg)	Wt (mg)	Inositol (μg)	Wt (mg)	Inositol (μg)
0	5*	83 ± 6†	161 ± 7	24 ± 2	55 ± 7	45 ± 4	149 ± 7	35 ± 3	66 ± 10	12 ± 1	7 ± 1
5	5	390 ± 29	1646 ± 255	107 ± 5	651 ± 63	151 ± 8	1209 ± 103	136 ± 15	354 ± 33		
10	5	974 ± 33	7233 ± 732	160 ± 7	957 ± 47	232 ± 17	1577 ± 100	374 ± 31	970 ± 81	49 ± 5	39 ± 8
20	5	1540 ± 108	11613 ± 1607	236 ± 4	1539 ± 38	391 ± 37	3471 ± 147	631 ± 34	1513 ± 134	76 ± 7	52 ± 7

* Mean wt 340 ± 79 g. † Mean ± S.E.

glands. The average concentration of *myo*-inositol in the accessory organs expressed as μg/g of tissue in sexually mature males (Group 8) was as follows: seminal vesicles 5544; coagulating glands 4397; dorsal prostates 6984; ventral prostates 1646 and coagulating glands 657. The concentrations of *myo*-inositol in the seminal vesicles, coagulating glands and dorsal prostates are higher than that reported for other tissues of the rat. For example, Mitchell and Isbell(13) have reported the following concentrations of inositol for various male rat organs expressed as μg/g liver 640; kidney 1300; spleen 1000; heart 650; lung 960; brain 1800 and muscle 220.

Marked decreases were observed in the weight and *myo*-inositol content of secretion-free male accessory organs following castration as shown by the data in Table III. The concentration of *myo*-inositol remained approximately the same in the seminal vesicles and ventral prostates but decreased in the coagulating glands, dorsal prostates and Cowper's glands. The *myo*-inositol concentration of the seminal vesicles and ventral prostates was higher 2 days following castration than in the normal animal indicating that the inositol content of these organs does not decrease as rapidly as does the organ weight.

Testosterone propionate (500 μg per day) was administered to groups of 20-day castrate males for periods of 5, 10 and 20 days. The data are presented in Table IV. The weights of male accessory glands and their *myo*-inositol content returned to normal following 10 days of hormone injection. However, after 20 days of treatment the weights of the seminal vesicles had increased 18 times, coagulating glands 10, dorsal prostates 8, ventral prostates 18 and Cowper's glands 6. During the same period the *myo*-inositol increased 72 times in the seminal vesicles, 28 in the coagulating glands, 23 in the dorsal prostates, 23 in the ventral prostates and 7 in the Cowper's glands. A comparison of the data obtained from castrates receiving androgen for 20 days (Table IV) with that from mature, intact males (Group 8, Table II) indicates that the weights and *myo*-inositol contents of the seminal vesicles, coagulating glands, dorsal prostates and ventral prostates of the hormone treated

TABLE V. Effect of Androgen and Feeding of *Myo*-inositol on *Myo*-inositol Concentration in Seminal Vesicle and Dorsal Prostate Tissues of Castrate Rats.

Animal wt (g)	Animals per group	Treatment†		Seminal vesicles		Dorsal prostates	
		Testosterone propionate	Inositol	Wt (mg)	Inositol (μg/g)	Wt (mg)	Inositol (μg/g)
389 ± 4*	9	—	—	63 ± 1*	1570 ± 133*	31 ± 2*	2058 ± 162*
388 ± 10	9	—	+	61 ± 3	1705 ± 148	30 ± 2	2439 ± 188
376 ± 6	9	+	—	148 ± 9	1394 ± 110	70 ± 3	2591 ± 199
406 ± 6	9	+	+	155 ± 7	1606 ± 95	72 ± 5	3489 ± 227

* Mean ± S.E.

† Testosterone propionate, 500 μg, 60, 36 and 12 hr before killing; *myo*-inositol, 25 mg/100 g wt, 12, 8 and 4 hr before killing.

animals were greater than those of the intact males.

The *myo*-inositol present in male accessory glands may be synthesized by these organs as well as selectively accumulated from the blood. In the latter case, it may be produced by bacterial action in the alimentary tract or synthesized in other organs. Halliday and Anderson (11) isolated *myo*-inositol-C^{14} from carcasses of rats which had received injections of glucose-1-C^{14}. Data on the selective uptake of *myo*-inositol by the seminal vesicles and dorsal prostates of castrate animals are presented in Table V. The *myo*-inositol contents of the seminal vesicles and dorsal prostates of the *myo*-inositol fed group were not significantly different from the control group not receiving this substance. However, when similar *myo*-inositol fed animals were given androgen, the dorsal prostates showed a significant increase ($P = 0.01$) in concentration. There was no significant increase in *myo*-inositol concentration in the seminal vesicles of hormone treated animals receiving *myo*-inositol. It is of interest that Gunn *et al.*(14) have observed that the dorsolateral prostate of the rat concentrates Zn^{65} from 15 to 25 times more than any other organ.

Summary. The *myo*-inositol content of male accessory organs was determined in growing, adult, castrate and androgen treated castrate rats. The *myo*-inositol concentration (μg/g/gland) was 5544 in the seminal vesicles, 4397 in coagulating glands, 6984 in dorsal prostates, 1646 in ventral prostates and 657 in Cowper's glands of mature animals. The *myo*-inositol content of the male accessory organs is dependent upon androgen. This relationship may be either a direct effect of male hormone on *myo*-inositol synthesis or an indirect response resulting from its action upon the total activity of the male accessory organs. It was demonstrated that the dorsal prostates can selectively accumulate *myo*-inositol in androgen treated castrate rats.

1. Scherer, J., *Ann. der Chem. und Pharm.*, 1850, v73, 322.

2. Eastcott, E. V., *J. Phys. Chem.*, 1928, v32, 1094.

3. Woolley, D. W., *J. Biol. Chem.*, 1941, v139, 29.

4. Hamilton, J. W., and Hogan, A. G., *J. Nutr.*, 1944, v27, 213.

5. Gavin, G., and McHenry, E. W., *J. Biol. Chem.*, 1941, v139, 485.

6. Eagle, H., Oyama, V. I., Levy, M., and Freeman, A., *Science*, 1956, v123, 845.

7. Geyer, R. P., and Chang, R. S., Proc. Soc. Exp. Biol. and Med., 1957, v95, 315.

8. Mann, T., *Proc. Roy. Soc.* (*London*) Series B, 1954, v142, 21.

9. Stetten, M. R., and Stetten, D., Jr., *J. Biol. Chem.*, 1946, v164, 85.

10. Daughaday, W. H., Larner, J., and Hartnett, C., *ibid.*, 1955, v212, 869.

11. Halliday, J., and Anderson, L., *ibid.*, 1955, v217, 797.

12. Beadle, G. W., *ibid.*, 1944, v156, 683.

13. Mitchell, H. K., and Isbell, E. R., *Univ. of Texas Publ.* 1942, no. 4237, 37.

14. Gunn, S. A., Gould, T. C., Ginori, S. S., and Morse, J. G., Proc. Soc. Exp. Biol. and Med., 1955, v88, 556.

Received September 3, 1957. P.S.E.B.M., 1957, v96.

Isolation of Cortisol from Guinea Pig Bile.* (23493)

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Cortisol has been found to be the major adrenal hormone in the plasma and urine of the guinea pig(1,2). Wyngaarden *et al.*(3) find a considerable amount of chloroform-soluble compounds containing C^{14} in the bile from guinea pigs subsequent to the administration of cortisol-4-C^{14}. In view of the recent isolation of tetrahydrocortisol and tetrahydrocorticosterone from cattle bile after hydrolysis with β-glucuronidase(4) it seemed worthwhile to determine the nature of the metabolites in guinea pig bile. The present study deals with the isolation of cortisol in plasma and bile from guinea pigs under ACTH stimulation.

Methods. Normal albino guinea pigs weighing between 250-350 g were used. The animals were given 3 I.U. of ACTH in gel per 100 g body weight subcutaneously 6 hours before sampling of blood and bile. The control animals were handled in the same fashion as those given ACTH except for the administration of the tropic hormone. To another group of animals 1 mg/100 g body weight of an alkali-extracted polysaccharide complex from *K. pneumoniae* was given intravenously on 3 successive days, and at different time intervals after administration of the third dose blood and bile were collected. For the collection of blood and bile, the animals were sacrificed by clamping the neck with a forceps. This method gives lower levels of plasma 17-hydroxycorticosteroids (17-OHCS) than those seen in animals acutely anesthetized with ether(5). Plasma and bile samples were obtained from the same animal, extracted with methylene chloride, and processed as described by Eik-Nes(6). From 0.5-4 ml of plasma and from 0.1-1 ml of bile were used per determination. These volumes were made up to 10 ml with water before extraction. Only samples exhibiting a typical Porter-Silber chromogen with a higher absorbancy at 410 mμ than at either 390 or 430 mμ are included in this publication. Values of 17-OHCS were corrected on the basis of the recovery of 0.5 γ cortisol taken through the total procedure.

Results. When plasma levels of 17-OHCS in the guinea pig are elevated by ACTH stimulation, free 17-OHCS appears in the bile (Table I). Under the experimental conditions used, we failed to detect a typical Porter-Silber chromogen in the bile of "normal" guinea pigs. Animals exposed to the "stressful" environment associated with the administration of bacterial polysaccharide exhibit free 17-OHCS in the bile as soon as the plasma concentration of these steroids is elevated (Fig. 1). Free 17-OHCS seem to persist in the bile after the plasma levels have returned to the pre-stressed range.

To determine whether the typical Porter and Silber chromogen in plasma and bile was cortisol(7), the following experiment was done: Twenty-two guinea pigs were sacrificed 6 hours after subcutaneous administration of 3 I.U. ACTH per 100 g of body weight. From this group of animals a total of 10 ml of bile and 50 ml of blood plasma was obtained. Plasma and bile were extracted with methylene chloride, partitioned between benzene and water and the water phase re-extracted with methylene chloride. The methylene chloride extracts were evaporated under nitrogen. The Porter-Silber reaction was done on an aliquot of each extract and a typical chromogen found(8). Using cortisol as reference standard, the pooled bile sample contained 400 γ of 17-OHCS per 100 ml and the pooled plasma sample, 140 γ per 100 ml.

The remaining portions were subjected to paper chromatography using the formamide-chloroform system of Zaffaroni and Burton

* This investigation was supported in part by research grants from Natl. Cancer Inst., N.I.H., U.S.P.H.S., Bethesda, Md.

(9). Only one compound was found in plasma and bile; this compound showed the same mobility on paper as authentic cortisol and could be detected by the ultraviolet scanning technic(10). The paper strips were eluted and the concentrated eluates read in the ultraviolet part of the spectrum using a Beckman DK 2 Spectrophotometer. Maximal absorbancy was found at 241 mμ for both the plasma and bile eluates. Acetylation with radioactive acetic anhydride in pyridine was then performed(11). In both the plasma and the bile extracts a radioactive compound moving chromatographically like the acetate of cortisol was detected. Finally, the acetate of the compound in bile and plasma was oxidized with chromic trioxide. After oxidation the compound was rechromatographed and behaved now like cortisone acetate in the chloroform-formamide system of Zaffaroni and Burton.

Discussion. The data show that high levels of cortisol in the blood plasma of guinea pigs are associated with the presence of this steroid as a free alcohol in the animal's bile. The normal guinea pig excretes a large amount of cortisol in the urine(2) and the conjugated metabolites of adrenal steroids in guinea pig urine are not glucuronides(2) as in man(12). A radioactive chloroform-extractable compound has been found in guinea pig bile following the administration of about 100 γ cortisol-4-C^{14}(4); 60% of the total radioactivity in the bile from guinea pigs given cortisol-4-C^{14} was, however, conjugated, but probably not in glucuronide form. Conjugation by the liver, therefore, occurs in this animal species, and one may presume that reduction of the steroid nucleus precedes the conjugation. In man, hydroxylation in position 3 of the adrenal steroids has been reported to be the rate limiting step in their metabolism(13). The presence of cortisol in guinea pig bile at a time when the plasma concentration of this steroid is high, could indicate either that enzymatic reduction occurs at a very slow speed or that the high concentration of cortisol passing through the animal's liver exceeds the capacity of the enzymes.

TABLE I. Levels of 17-OHCS in Plasma and Bile from the Same Guinea Pig

Condition	17-OHCS γ/100 ml Plasma	Bile
Normal	20	0*
	31	0*
	10	0*
	43	0*
	17	0*
	22	0*
6 hr after ACTH	144	260
	209	315
	215	310
	212	375
	189	301
	210	298

* No detectable Porter-Silber chromogen.

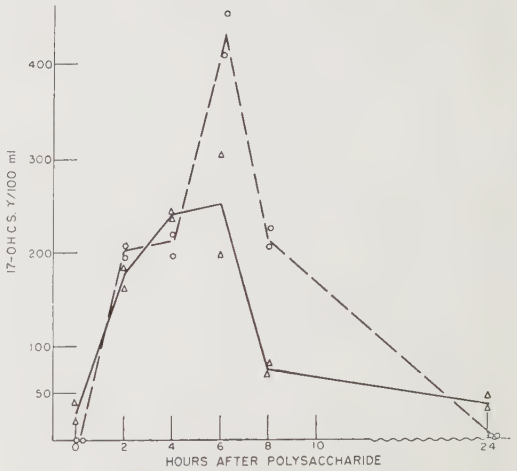

FIG. 1. △ = Plasma levels of 17-OHCS in individual guinea pigs sacrificed at different time intervals following intravenous administration of 1 mg *K. pneumoniae* polysaccharide complex/100 g body weight. ○ = Bile levels of 17-OHCS in the same animals. The average bile and plasma curves for 17-OHCS are plotted. All animals had received 1 mg *K. pneumoniae* polysaccharide complex/100 g body weight intravenously on the 2 preceding days.

Summary. Free cortisol has been isolated in the bile from guinea pigs with high plasma concentrations of this steroid. A limited hepatic ability to hydroxylate this hormone in position 3 of its nucleus has been suggested as a possible cause.

1. Bush, I. E., *J. Physiol.,* 1951, v115, 12.

2. Burstein, S., and Dorfman, R. I., *J. Biol. Chem.,* 1954, v206, 607.

3. Wyngaarden, J. B., Peterson, R. E., and Wolff, A. R., *ibid.,* 1955, v212, 936.

4. Glick, J. H., *Endocrinology,* 1957, v60, 368.

5. Jones, R. S., and Eik-Nes, K., in preparation, 1957.

6. Eik-Nes, K., *J. Clin. End. and Metab.*, 1957, v17, 502.

7. Marks, L. J., and Leftin, J. H., *ibid.*, 1954, v14, 1263.

8. Porter, C. C., and Silber, R. H., *J. Biol. Chem.*, 1950, v185, 201.

9. Zaffaroni, A., and Burton, R. B., *ibid.*, 1951, v193, 749.

10. Haines, W. J., and Drake, N. A., *Fed. Proc.*, 1950, v9, 180.

11. Berliner, D. L., *ibid.*, 1956, v15, 219.

12. Buehler, H., Katzman, P. A., Doisy, P. P., and Doisy, E. A., PROC. SOC. EXP. BIOL. AND MED., 1949, v72, 297.

13. Peterson, R. E., Wyngaarden, J. B., Guerra, S. L., Brodie, B. B., and Bumin, B. B., *J. Clin. Invest.*, 1955, v34, 1779.

Received September 3, 1957. P.S.E.B.M., 1957, v96.

Transformation and Culture Phase Dissociation of Staphylococcus (*Micrococcus pyogenes*) (23494)

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A distinctive pattern of dissociation evolved from studies on *Gaffkya tetragena*. A specific immunologic white colony type gave rise to yellow, pink, pink-yellow and brown types, each with its mucoid, smooth and rough phases, and in addition a translucent form (1,2,3). The variants emerged best in aging cultures in broth or on agar and seemed to appear by chance. Because of a close relationship between *G. tetragena* and *Staphylococcus,* tests were made with similar methods to see if a parallel dissociative pattern pertained.

Methods. Cocci from a single yellow colony of *Staphylococcus PCI 209 P* (U.S. Food & Drug Admin.) served as the inoculum of 100 ml nutrient broth in flasks. After incubation at room temperature for 3 to 7 months, subcultures were made by streaking or as 5 or 6 point colonies on nutrient agar 7-10 mm thick in plates sealed with a strip of parafilm to delay drying. The plates were similarly aged and continually inspected for visible evidence of colonial changes.

Results. Point colonies made from a 54-day flask culture were of the smooth(S) yellow phase 10 mm wide after 20 days. At first they were of the usual yellow butter color and consistence, but later faded until some were yellow in the center shading into white; others turned cream-color or white. On further aging a sharply defined, indented, colorless, translucent rim appeared around some; others were studded or rimmed or both with minute yellow and white daughter colonies. The white ones usually were yellowish by transmitted light. Subcultures from the yellow, white and translucent areas, and from the yellow and white nubbins, with exceptions to be described, grew as the original S-yellow form as if youth and crowding had delayed the formation of pigment. Shades from nearly white, cream, buff, pale yellow (citreus), yellow (aureus), to deep orange (auranticus) appeared in many streaked cultures and point colonies for no discernible reason. Subculture usually reproduced the original yellow tint. Growth at 10°C or 37°C made little difference in color change. One set of cream-colored point colonies 90 days old and 22 mm broad were studded and edged with pale daughter colonies. These in turn were surrounded by a translucent band with another rim of minute colonies (Fig. 1).

Cocci from the S-yellow colonies were coagulase-positive, fermented mannitol, liquefied gelatin and hemolized blood.

Origin of the Mucoid-Yellow Phase. Subcultures from the pale areas of a 58-day S-yellow colony grew as thick, glistening, pale yellow colonies, confluent where crowded (Fig. 2) in contrast with the usual discrete S-

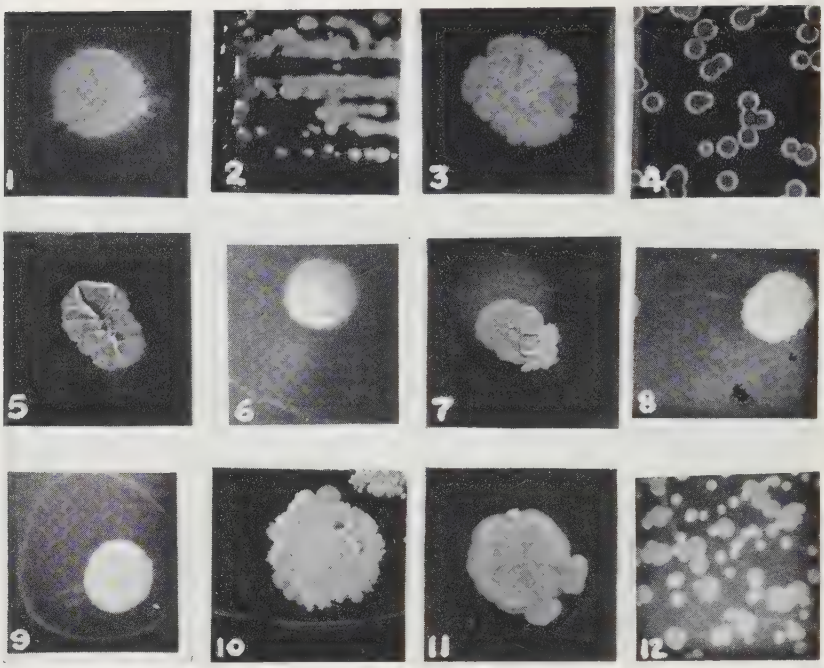

FIG. 1. S-yellow colony 90 days old studded and edged with daughter colonies surrounded by a translucent band with another rim of barely visible colonies.
FIG. 2. Confluent M-yellow colonies.
FIG. 3. M-yellow colony with sectors.
FIG. 4. Rimmed M-yellow colonies.
FIG. 5. R-yellow colony.
FIG. 6. R-yellow colony with a white daughter in the translucent rim.
FIG. 7. R-yellow colony with M-yellow sector in the middle and a white one to the right.
FIG. 8. S-white colony studded with yellow and white daughters.
FIG. 9. S-white colony.
FIG. 10. S-white colony, 2 mo. old studded with white papillas and with mucoid-white outgrowths.
FIG. 11. M-white colony with M-white and S-white outgrowths.
FIG. 12. M-white colonies and smaller S-white ones.
All colonies are in natural size.

yellow ones. Touched with a wire, they were tough or glairy and pulled like mucin. Further transfer on streaked plates bred true. Point colonies usually reverted to the S form or formed M colonies (Fig. 3), and a few grew as rough yellow colonies. Senile flask cultures of S-yellow usually changed to M-yellow. On occasion, colonies seemed to autolyze in the center but had a thick mucoid-yellow rim (Fig. 4). Subcultures from both areas bred true.

Cocci of the M colonies were smaller than the parent ones. They caused mucinous growth in broth, were unclustered, appeared at times to have capsules and often were enmeshed in a stringy pink-stained matrix, possibly of capsular substance. No swelling or clumping occurred when immersed in homologous antiserum. They were coagulase-positive, fermented mannitol, liquefied gelatin and were hemolytic.

Origin of the Rough-Yellow Phase. A 97-day flask culture of S-yellow cocci streaked on agar at first grew as homogenous cream-colored colonies. After a month, a few colonies were larger and less opaque. Both kinds on transfer grew as viscid, confluent M-yellow ones. On a point colony plate, cocci from the small colonies reproduced S-yellow ones, but those from the large ones grew into 10 mm, flat, yellow, lusterless, deeply crinkled and concentrically or radially ridged colonies, obviously the hitherto undescribed R-yellow phase (Fig. 5 and 6). When fished with a

wire, large, dry, crumbly portions lifted away. Each colony was surrounded later by a translucent fringe. As mentioned in the preceding section, M-yellow colonies at times gave rise to R-yellow ones.

In one month-old colony, a white daughter colony emerged at the tip of a ridge (Fig. 6). Subcultures of the R-yellow base gave M-yellow colonies. Subcultures from the white outgrowth at times reverted to mucoid-yellow colonies, but at others, formed different slightly ridged pale yellow colonies with a white rim. One colony in addition developed a large paler yellow sector and a white sector both of which were ridged (Fig. 7). Transfers from the paler yellow sector gave M-yellow colonies. From the white sector 2 kinds of white colonies grew, one as S-white, the other as a new form of high-domed glossy mucoid white.

Another point R-yellow colony was pale, slightly ridged and surrounded by a whitish rim in which a deep yellow sector grew after 3 weeks. Transfer from the pale yellow center gave typical R-yellow colonies on one occasion and M-white on another; the deep yellow rim sector was a reversion to the S-yellow phase.

Subcultures of R-yellow cocci on streaked plates usually grew as the M-phase; point colonies usually grew as R-yellow, but often gave rise to M or S yellows and to a mucoid white. Some point colonies and occasionally crowded ones less rough than the extremely rough ones may have represented intermediate forms. Evidently the R-yellow phase is less stable than the M or S.

R-yellow cocci were small, clustered but with no evidence of capsules or ropy growth and had biologic characteristics in common with the M and S ones.

Origin of a Smooth-White Type. As stated previously, the original senile S-yellow point colonies had yellow and white daughter ones (Fig. 8). Subcultures of both usually evolved into the S-yellow phase, but on several occasions those from the white daughters produced mostly yellow and a few white colonies. From a 7-month S-yellow flask culture, a streaked plate revealed almost all M-yellow colonies and a row of about 20 white ones. Point colonies from both sources grew more slowly than the yellow and formed smaller, white, porcelain-like, flat colonies 10 to 12 mm wide after a month or two (Fig. 9). They were regarded as the S-white phase. Subcultures were stable. Old colonies studded with white papillas had the deceptive appearance of roughness and had outgrowths of mucoid white (Fig. 10).

The cocci were usually much larger than those previously mentioned, had no capsule and many were in diploform and tetrads. Both the cocci and the colonies were morphologically indistinguishable from those of S-white *G. tetragena*. They were weakly hemolytic, coagulase-negative and did not ferment mannitol nor liquefy gelatin.

Origin of the Mucoid-White Phase. Subcultures from a 3-month flask seeded with S-white cocci grew as colonies different from the parent. On one occasion a mucoid white sector appeared in an M-yellow colony. Papillas on a 2-month S-white point colony (Fig. 10) and a sector of an R-yellow one (Fig. 7) also differed. The colonies therefrom were glistening, pearly-white and less opaque; later they flattened, became drier and developed sectors, areas and outgrowths of opaque white or of even more translucency (Fig. 11). Young colonies, touched with a wire were viscid and drew out in filaments; older ones, though drier, were more fluid than the S-white ones. They were confluent when crowded resembling those of Fig. 2. Numbers of small, dense, white, domed colonies appeared among the more translucent ones (Fig. 12) indicating reversion to S-white. Thus far, neither the M-white nor the S-white reverted to the yellow type and, as with *G. tetragena,* no R-white form emerged.

Cocci of the M-white phase were clustered, often in tetrads, seemed to have less prominent capsules than the M-yellow, often were enmeshed in pink stained material, were larger than the yellow ones. Growth in broth was slimy. The biologic reactions were those of the S-white.

Translucent forms. Subcultures of a white daughter colony from a giant S-yellow one grew into crowded yellow colonies after 2

days and scattered about were minute, flat, thin, barely visible, discreet and confluent, colorless, translucent ones. On only a few other occasions did similar translucent colonies appear among S-yellow ones. They were suspected as being the anticipated translucent form or the so-called G form. Some of them were immature yellow ones delayed in development by crowding because subcultures usually gave rise to yellow colonies. Subcultures on agar or in broth at times bred true, grew slowly or not at all and some died. Point colonies seldom exceeded 2 or 3 mm in width and daughter colonies formed before growth ceased. On no occasion did evidence of reversion to any other form appear as was often the case with *G. tetragena*. For that reason the question of contamination was not excluded. The cocci were small, many in tetrads and clumps, pleomorphic and polychromatic similar to those of the G or translucent form described for other bacteria. They were coagulase-negative, did not liquefy gelation, ferment mannitol nor hemolyze blood.

Small Colony Variants. These forms described by others seldom were seen. Small ones often were small because they were crowded. A few small yellow and white colonies occasionally remained small on subculture but usually grew into large ones when transferred. Growth in penicillin-broth did not induce their appearance better than aging of cultures.

Comment. Previous investigations of the dissociative pattern of *Staphylococcus* did not reveal the classic M, S, R changes as established for *G. tetragena*(3), *Pneumococcus*(4) or *Streptococcus*(5). Pinner and Voldrich described what now appears to be type transformation of the yellow (aureus) into the lemon-yellow (citreus), white (albus), and pink (roseus) forms, but saw no R forms and M forms were not mentioned(6). The citreus forms were stable, but the albus reverted to the aureus after growth in homologous antiserum, and the roseus to the albus after inoculation into animals. They regarded color transformation as analogous to phase variation of other bacteria. Color-type transformation was similar to that I later observed in *G. tetragena*.

Bigger(7) described a scheme difficult to reconcile with the usual pattern. A number of aureus and albus variants were classified as smooth with varying degrees of viscidity and as rough viscid forms. Some sticky ones which pulled out like glue probably were mucoid forms. Photographs of his 3-day-old rough colonies bear no resemblance to the R form here described.

Staphylococcus aureus of all strains tested by Hoffstadt and Youmans gave rise to white and G colonial forms(8). They described 8 kinds called rough, but some were translucent bluish-white, and photographs again do not resemble the R colonies described here. These investigators and Swingle(9) observed small colony variants (G form), and others(10,11) obtained them by growth in broth containing penicillin. They usually reverted to the parent form on subculture. Similar small colonies appeared occasionally in my experiments but not more often after exposure to penicillin than after aging.

The studies of these investigators and the ones presented here indicate that dissociation of *Staphylococcus* like that of other bacteria is more complicated than the simple, visibly or serologically detectable type and type-color transformation and respective M, S, R phase variation. Thus far the simplified scheme appears as:

Mucoid yellow	Mucoid white
Smooth yellow	Smooth white
Rough yellow	

It is the counterpart of the scheme depicted for other Gram-positive pathogenic cocci, namely, *G. tetragena, Streptococcus,* and *Pneumococcus*. For the latter it is:

Type I	Type II	Type III	
Mucoid	Mucoid	Mucoid	etc.
Smooth	Smooth	Smooth	
Rough	Rough	Rough	

Conversion of pneumococci of one type into another was induced by growth of M-pneumococci or of R-pneumococci derived from any type in animals or in broth with dead M cocci containing deoxyribonucleate of the donor cocci(12,13). Type transmutation of *G. tetragena* and of *Staphylococcus* was spon-

taneous. Strains established as aureus, albus and roseus probably are variant forms of a common ancestor.

Granting an analogy of dissociative patterns, one may predict an R-white phase, a pink type as Pinner found, and perhaps others for *Staphylococcus*. Effort to educe them is under way. It would be of importance to know the significance of the mucinous substance of the M phase, whether the yellow and white staphylococci are type specific by serologic or 'phage methods and if both forms convert to different respective serologic yellow and white types.

Staphylococcus and *G. tetragena* seem to be much more closely related than generally thought. The resemblance in colonial appearance, and in coccal size, shape, staining and arrangement probably often leads to bacteriologic diagnostic error. It is impossible to differentiate the respective S-yellow or S-white forms of either on that basis. The biologic differences as listed(14) are minor and may vary even among identical strains; and they differ from some reported here. Officially, *St. albus* liquefies gelatin and ferments mannitol; my white forms did not. *G. tetragena* is said not to liquefy gelatin nor ferment mannitol, in agreement with my tests(1), but the myth of pathogenicity for mice is perpetuated. In my hands, growth of staphylococci and of tetragenas was inhibited by similar amounts of penicillin, the M cocci of both made a mucinous substance and the yellow forms grew more luxuriantly and resisted heat better than the white forms.

Summary. A partial pattern of dissociation into color-type and-phase variation emerged after aging a strain of *Staphylococcus*. From the smooth yellow (aureus), a mucoid yellow and a rough yellow colony form were derived, and in addition a smooth white type (albus) and its mucoid white phase. Thus far the dissociative pattern is analogous with that of *G. tetragena* and *Pneumococcus* as regards type and phase variation and interconvertibility. It may apply to *Staphylococcus* in general.

1. Reimann, H. A., *J. Bact.*, 1936, v31, 385.
2. ———, *ibid.*, 1937, v33, 499.
3. ———, *ibid.*, 1937, v33, 513.
4. Dawson, M. H., *J. Path. and Bact.*, 1934, v39, 323.
5. Dawson, M. H., Hobby, G. L., and Olmstead, M., *J. Inf. Dis.*, 1938, v62, 138.
6. Pinner, M., and Voldrich, M., *ibid.*, 1932, v50, 185.
7. Bigger, J. W., Boland, C. R., and O'Meara, R. A. Q., *J. Path. and Bact.*, 1927, v30, 261.
8. Hoffstadt, R. E., and Youmans, G. P., *J. Bact.*, 1932, v51, 216; 1934, v27, 551.
9. Swingle, E. L., *ibid.*, 1935, v29, 1935.
10. Wise, R. I., *Ann. N. Y. Acad. Sci.*, 1956, v65, 169.
11. Youmans, G. P., PROC. SOC. EXP. BIOL. AND MED., 1945, v58, 56.
12. Reimann, H. A., *J. Exp. Med.*, 1929, v49, 237.
13. Dawson, M. H., and Sia, R. H. P., *ibid.*, 1931, v54, 681.
14. Breed, R. S., Murray, E. G. D., and Hitchens, A. P., *Bergey's Manual of Determinative Bacteriology*, 6th Ed., Williams & Wilkins Co., Balto., 1948.

Received September 4, 1957. P.S.E.B.M., 1957, v96.

Increased Sensitivity to Estrogen in Uterine Hyperplasia in DBA x CE and Reciprocal Hybrid Mice.* (23495)

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At 6 to 7 months of age, virgin female DBA x CE and reciprocal hybrid mice spontaneously develop a progressively severe endocrine imbalance involving the ovaries, adrenal cortex and pituitary(1,2). One of the more prominent morphological features of this syndrome is marked enlargement of the uterus

* This investigation was supported by research grants from Natl. Cancer Inst., N.I.H., P.H.S., and by a grant-in-aid from American Cancer Society.

TABLE I. Uterine Response to Estrogen in Ovariectomized DBA × CE and Reciprocal Hybrid Mice in Relation to Age.

Age (mo)	No. of mice	Treatment	Body wt (g) ± S.E.	Uterine wt (mg) ± S.E.		Uterine wt/body wt (mg/g) ± S.E.	
4–5	10		26.2 ± .5	29.1 ± 1.1	(340% incr.)	1.1 ± .0	(336% incr.)
	11	Est	26.5 ± .7	98.8 ± 6.7		3.7 ± .3	
9–12	11		29.3 ± .6	40.2 ± 2.7	(449% ")	1.3 ± .1	(469% ")
	10	Est	29.3 ± .7	180.5 ± 23.5		6.1 ± .3	
15–18	10		32.2 ± .4	72.1 ± 12.2	(498% ")	2.5 ± .1	(464% ")
	12	Est	31.1 ± .7	358.8 ± 11.2		11.6 ± .5	

accompanied by muscular and glandular hyperplasia, adenomyosis, generalized fibrosis and vascular congestion. Previous studies have demonstrated that these uterine lesions result, at least in part, from gradual prolongation of the estrogenic phases of the estrous cycle which culminates in a condition approaching "constant estrus" by 15 months of age(3,4). The question has arisen, however, whether the hyperplasia of the uterus might also be due in part to an increase in sensitivity of this organ to estrogen in addition to increased estrogenic stimulation previously demonstrated. The present experiment was designed to test this hypothesis.

Procedure. Sixty-four virgin female mice, offspring of DBA x CE and reciprocally crossed parents, were used. The mice were born in the Jackson Laboratory colony and were maintained at a temperature of 70°F on a diet of Purina Laboratory Chow and water *ad libitum.* At the beginning of the experiment animals in 3 age groups were selected with reference to the development and progress of the hyperovarian syndrome: (1) 4 to 5 months of age—prior to appearance of symptoms, (2) 9 to 12 months—during most rapid progress of the syndrome, and (3) 15 to 18 months—after the syndrome is well advanced. Uterine sensitivity to estrogen was determined in the following manner. The mice were bilaterally ovariectomized. Fourteen days later approximately one-half of the animals in each age group were sacrificed without further treatment. The remaining animals were injected subcutaneously with 3 μg of crystalline estradiol diproprionate in 0.1 ml of sesame oil and were sacrificed 48 hours later. The uteri of both control and treated mice were removed by cutting across the cervix and trimming the cornua free of their attachment to the mesometria. The cornua were slit and blotted with filter paper to remove any fluid in the lumina. Uterine weights were then determined to the nearest 0.1 mg with a Roller-Smith torsion balance. The estrogen response was calculated as the percentage of mean weight increase of the uteri of injected animals compared with mean weight in untreated controls of the corresponding age group.

The *results* are summarized in Table I. The increment in uterine weight following injection of the standard test dose of estradiol diproprionate in mice ovariectomized at 4 to 5 months of age (*i.e.*, prior to the onset of uterine hyperplasia) was approximately 340%. In mice ovariectomized and tested after the appearance of uterine hyperplasia, on the other hand, the uterine weight response was 449% at 9 to 12 months and 498% at 15 to 18 months.

It can be seen from the Table that with increasing age there is a considerable increase both in body weight and in post-ovariectomy uterine weight in untreated controls as well as in injected animals. If the actual mean uterine weights are expressed in proportion to mean body weights of the animals in each group, the uterine response to estrogen is 336% in pre-syndromic mice and 469% and 464% respectively in the 2 groups exhibiting the syndrome. When calculations are made in this manner, the apparent difference in responsiveness between the 2 latter groups disappears.

Discussion. The present observations clearly demonstrate that in DBA x CE and

reciprocal hybrid mice a considerable increase in uterine sensitivity to estrogen develops in conjunction with the hyperovarian syndrome. The observed increment represents a minimal difference because of the experimental procedure followed. That is, whereas the body weights of mice in the 9 to 12 and 15 to 18-month-old groups were about 11% and 20% greater than in the youngest group of animals, the test dose of estrogen was the same in all instances. It is probable that if the amount of hormone given had been increased in proportion to gain in body weight, the increment in uterine responsiveness in the older mice would have been even greater than that actually measured. In view of the present findings, then, it seems reasonable to conclude that the uterine hyperplasia which develops in these hybrid mice after onset of the hyperovarian syndrome is due in part to increased responsiveness of the uterus to estrogen as well as to the hyperestrinism previously reported.

The present findings also have implications of general interest apart from their bearing on the pathogenesis of uterine lesions peculiar to these particular mice. In endocrine studies cognizance is seldom taken of possible effects of aging on hormone sensitivity of target organs due, no doubt, to the paucity of information on the subject. In so far as the reproductive tract is concerned, the only extensive studies have been those of Hooker(5) and Price and Ortiz(6) who have demonstrated a temporary increase of 500% or more in hormone sensitivity during puberty in the rat. Unfortunately their observations included but a relatively short period after puberty. In a small number of ovariectomized monkeys, on the other hand, Allen and his co-workers(7) found that only one-third to one-half as much estrone was needed to induce withdrawal bleeding from uteri of mature animals as compared with immature and puberal animals. The present observations and those cited above agree that age-related changes in hormone sensitivity of the reproductive tract occur. Due to different experimental conditions and species studied, however, no clear-cut pattern can be discerned. It is evident that further investigation of this phenomenon is imperative, especially for the proper evaluation of data based on bioassay technics and for elucidation of endocrine-related pathologic conditions of the uterus such as liomyomatosis and endometrial hyperplasia which occur in women with increasing frequency as the menopause is approached.

Summary. Virgin female DBA x CE and reciprocally crossed hybrid mice were ovariectomized at 4 to 5, 9 to 12, and 15 to 18 months of age. Two weeks later one-half of the animals in each group were injected with 3 μg of estradiol diproprionate and were sacrificed 48 hours later. The uterine response was calculated by comparing uterine weights (adjusted to mg/g body weight) after estrogen treatment with those in the untreated controls. In the 4 to 5-month-old animals the increment in uterine weight was 336%; in the 2 older groups, 469% and 464% respectively. These data indicate that the uterine hyperplasia which develops spontaneously in these mice after 7 to 8 months of age is due in part to increased uterine sensitivity to estrogen as well as to the hyperestrinism previously known to occur.

1. Christy, N. P., Dickie, M. M., Atkinson, W. B., and Woolley, G. W., *Cancer Research,* 1951, v11, 413.

2. Dickie, M. M., and Woolley, G. W., *ibid.,* 1949, v9, 372.

3. Atkinson, W. B., and Dickie, M. M., *ibid.,* 1953, v13, 165.

4. Dickie, M. M., Atkinson, W. B., and Fekete, E., *Anat. Rec.,* 1957, v127, 187.

5. Hooker, C. W., *Endocrinol.,* 1942, v30, 77.

6. Price, D., and Ortiz, E., *ibid.,* 1944, v34, 215.

7. Allen, E., Diddle, A. W., Burford, T. H., and Gardner, W. U., *Am. J. Physiol.,* 1936, v117, 381.

Received September 9, 1957. P.S.E.B.M., 1957, v96.

Bleeding Syndrome in Thrombin-Injected Rats Deficient in Fibrinogen and Antihemophilic Factor. (23496)

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Hemostatic mechanisms may be studied conveniently in rats by microscopic observation of the platelet plugs formed after incising mesenteric arteries or veins(1). In rats made hypofibrinogenemic by the intraperitoneal injection of thrombin(2), platelet plugs either failed to form or were ineffective in arresting bleeding(3). Since fibrin is not histologically demonstrable in the plugs formed in the mesenteric blood vessels of normal rats(3), it seemed unlikely that the low level of fibrinogen was responsible for the defective hemostasis. We, therefore, undertook a more complete study of the clotting factors in thrombin-injected rats to determine whether other deficiencies were present which could account for the abnormal bleeding.

Methods. Male albino rats weighing between 75 and 130 g were used. Twenty-five hundred units of thrombin† (140 mg in 0.37 ml) were injected intraperitoneally into 17 unanesthetized rats. Nine of these were studied one hour after injection and 8, 2 hours after injection. Three control rats received an intraperitoneal injection of 140 mg of bovine albumin and the remaining 17 controls were not injected. After intramuscular administration of nembutal (4 mg/100 g), an exposed mesenteric vein was cut with sharp scissors, and observed for the formation of a platelet plug as previously described(1). Blood was then drawn from the abdominal aorta with a syringe and needle. Prothrombin time was measured by a modification of Quick's method(4). Prothrombin, factor V and factor VII concentrations were determined by modifications of Owren's methods (4). Prothrombin consumption was estimated by measuring the residual prothrombin in serum from blood oxalated after one hour at 37°C(4). Antihemophilic factor (AHF) and plasma thromboplastin component (PTC) were estimated from the amount of prothrombin remaining one hour after recalcifying a mixture of citrated hemophilic or PTC-deficient plasma and a 1:10 dilution of rat plasma(4). Fibrinogen was determined by Quick's modification of the Cullen and Van Slyke method, adapted for the use of 0.5 ml of plasma. Fibrinolytic activity at 37°C was estimated from the lysis time of clotted whole blood and of a mixture of bovine fibrinogen and thrombin with the rat's plasma euglobulin fraction(5). Platelet counts were performed by the direct method(6). Capillary fragility was measured on abdominal skin by Dalldorf's negative pressure method(7).

Results. Platelet plugs formed and bleeding ceased completely between 28 and 140 seconds after incising a mesenteric vein in 8 control rats. The plugs adhered to the vessel and could not easily be removed or fragmented with a forceps. If bleeding was renewed by such manipulation, hemostasis was reestablished more rapidly than after the initial incision.

In all of 11 thrombin-injected rats, bleeding continued for from 5 to 8 minutes before the vein was clamped to avoid exsanguination. Platelet plugs formed in only 3 of these animals (2 rats in the 1-hour group and 1 in the 2-hour group). They were not effective in arresting bleeding and could be readily dislodged or fragmented. Vasoconstriction, observed at the ends of the severed vein in all rats, did not appear to reduce blood flow appreciably, an observation which is in agreement with Hugues' results(8).

Gross autopsy findings on 16 animals which received thrombin included nasal bleeding in 2 and bloody urine in 6. Thymic lymph nodes were hemorrhagic in 8 animals, mesenteric nodes in 2. Two rats had ecchymoses in the diaphragm. No evidence of hemorrhage was

* Aided by grants from Am. Heart Assn. and the U. S. Army.

† Parke-Davis Bovine Topical Thrombin, lot No. 015043-C.

TABLE I. Changes in Clotting Factors One and 2 Hours after Intraperitoneal Injection of 2500 Units of Thrombin.

	Clot formed, No. rats	Lysis time, min.	Fibrinogen, mg %	Prothr. time, sec.	Prothr. conc., sec.	Factor V, sec.	Factor VII, sec.	Serum prothr., sec.	Platelets, $10^6/mm^3$	AHF, sec.
Controls	4 (4)	>1440 (4)	170–238 (4)	13.6 ± 3.4 (16)	21.3 ± 2.7 (16)	21.2 ± 3.9 (16)	24.9 ± 3.8 (16)	68.7 ± 23.3 (9)	1.07 ± .72 (9)	71.0 ± 30.2‡ (7)
1 hr	3 (6)	>1440 (3)	65–211 (6)	20.2–22.6 (3)	25.2 ± 3.1* (8)	29.1 ± 6.2* (8)	34.8 ± 4.5* (8)	45.0 ± 15.7† (9)	.71 ± .16* (9)	21.9 ± 5.3* (5)
2 "	2 (8)	40, 70 (2)	30 (1)	38, ∞ (2)	27.1 ± 4.6* (7)	24.8 ± 6.5 (7)	32.1 ± 4.6* (7)	30.6 ± 7.1† (3)	.43 ± .09* (6)	32.1 ± 8.4* (8)

Avg ± stand. dev. No. of animals given in parentheses.

* Compared with control value, $P < 0.01$. † Compared with control value, $P > 0.02$, < 0.05. ‡ The large stand. dev. is the result of one value of 29 sec. All other values were over 60 sec.

observed in the control animals.

No petechiae formed at a negative pressure of 30 cm Hg in 5 rats tested 2 hours after thrombin injection. However, local subcutaneous hemorrhages were observed in 3 of the injected rats, but in none of the controls.

Determinations of clotting factors are shown in Table I. Because of the small volume of blood available, it was not possible to do all determinations on every animal. Whole blood clotted in 5 thrombin-treated animals and the clot lysed rapidly in 2 of them. Lack of clot formation in 9 rats could be attributed in at least one instance to hypofibrinogenemia; in other instances no clot was noted even when fibrinogen concentration was as high as 85 mg%. This apparent failure of clot formation may have resulted from rapid lysis of small clots. It could not be ascribed to the presence of a potent circulating anticoagulant since clots were produced promptly after addition of purified human fibrinogen to the otherwise incoagulable blood from 5 of these rats. Fibrinolytic activity of the euglobulin fraction of plasma varied from 3 to over 24 hours in both control and test animals, and showed no correlation with whole clot lysis time.

No clots formed in prothrombin time determinations on 8 animals whose whole blood failed to clot. Among the rats whose whole blood clotted, the long prothrombin times of the 3 rats in the 1-hour group can be explained by the decreased concentration of prothrombin and Factors V and VII. The still longer prothrombin times of the 2 rats which showed whole clot lysis were not the result of unusually low concentrations of these factors, and may indicate that fibrinogenolysis had occurred during the brief interval between measurement of whole blood clotting time and prothrombin time.

Residual prothrombin in serum was significantly higher than normal after injection of thrombin. Platelet count decreased, but severe thrombocytopenia did not occur. Antihemophilic factor (AHF) was very much diminished. One hour after thrombin injection, a 1:10 dilution of rat plasma had no greater effect on the prothrombin consumption of he-

mophilic plasma than did saline so that AHF concentration was approximately zero. Some AHF was present in the plasma of rats 2 hours after thrombin injection, but the concentration was still significantly below normal. On the other hand, concentration of plasma thromboplastin component (PTC) was not measurably reduced by thrombin administration.

Discussion. Hypofibrinogenemia which occurs after intravenous injection of thrombin is believed to result from intravascular deposition of fibrin(9,10). Defibrination presumably also occurs in rats injected intraperitoneally with thrombin and probably accounts for the absence of clot formation in many of the animals. No anticoagulant was detected in the 5 rats tested. The occurrence of whole clot lysis in 2 animals suggests that fibrinolytic activity may have been partially responsible for lack of clot formation and reduction in fibrinogen concentration in some rats. Whether fibrinolytic activity resulted from activation of the rat's own plasminogen or of the bovine plasminogen present as a contaminant in the injected thrombin(11) was not determined. The fibrinolytic activity of the euglobulin fraction did not correlate well with the presence of whole clot lysis.

Tissue thromboplastin used during preparation of the thrombin is still present in the final product. Its injection could account for the fall in prothrombin(12,13), factor V(13), and perhaps factor VII. Factor V is activated by thrombin(14), and in the rat, active factor V, or serum accelerator globulin, is rapidly destroyed(15). This may contribute to the decrease in factor V. No greater depression of clotting factors was observed in the rats with whole clot lysis than in those whose clots did not lyse. Consequently, although prothrombin, factor V and factor VII, as well as AHF, can be destroyed by fibrinolysin(16) this enzyme did not seem to be important in diminishing their concentration in these experiments.

High serum concentration of prothrombin was found after thrombin injection. The magnitude of the defect in prothrombin consumption is even greater than is indicated by the data on serum prothrombin alone, since in the thrombin-injected rats, amount of prothrombin present in the plasma was low. Vandenbroucke and Verwilghen observed a decrease in prothrombin consumption after intravenous injection of thrombin in dogs, and attributed it to a defect in thromboplastin formation (17). From our data, it seems evident that deficiency of AHF is responsible for the poor prothrombin consumption. The low level of AHF is not surprising, since others have shown that AHF is inactivated by thrombin, both *in vitro* and *in vivo*(18,19).

The number of abnormalities in the clotting mechanism makes it difficult to determine the cause of the failure in platelet plug formation in thrombin-injected animals. However, the decreases in prothrombin, factor V and factor VII do not seem sufficiently great to prevent platelet plug formation(3). The absence of demonstrable fibrin in normal mesenteric plugs(3) makes it unlikely that the hemostatic defect is the result of the low concentration of fibrinogen or of the occasionally observed fibrinolytic activity. Since the tissue thromboplastin liberated when an exposed mesenteric vein is cut must be small in amount and rapidly washed away, it is possible that the formation of platelet plugs under these circumstances depends upon the formation of blood thromboplastin, for which AHF is required(20).

Summary. One and 2 hours after intraperitoneal injection of 2500 units of thrombin into rats, bleeding from severed mesenteric blood vessels was not arrested and platelet plugs rarely formed. Other manifestations of hemorrhagic tendency were present. Capillary resistance was not diminished. Fibrinogen concentration was low; whole clot fibrinolysis was occasionally observed; prothrombin, factor V, factor VII and platelets were somewhat reduced; PTC concentration was normal; and AHF concentration approached zero. It is suggested that the defect in hemostasis is predominantly the result of AHF deficiency.

1. Zucker, M. B., *Am. J. Physiol.*, 1947, v148, 275.
2. Warner, E. D., Brinkhous, K. M., Seegers, W. H., and Smith, H. P., PROC. SOC. EXP. BIOL. AND MED., 1939, v41, 655.

3. Zucker, M. B., *Blood Clotting and Allied Problems,* Trans. of Fourth Conference, Ed. J. E. Flynn, Josiah Macy, Jr. Fn., N. Y., 1951, pp149-152.

4. Lewis, J. H., Ferguson, J. H., Fresh, J. W., and Zucker, M. B., *J. Lab. Clin. Med.,* 1957, v49, 211.

5. Cliffton, E. E., and Cannamela, D., *J. Appl. Physiol.,* 1953, v6, 42.

6. Brecher, G., Schneiderman, M., and Cronkite, E. P., *Am. J. Clin. Path.,* 1953, v23, 15.

7. Dalldorf, G., *J. Exp. Med.,* 1931, v53, 289.

8. Hugues, J., *Arch. Internat. Physiol.,* 1953, v61, 565.

9. Jurgens, R., and Studer, A., *Helv. Physiol. et Pharmacol. Acta,* 1948, v6, 130.

10. Nolf, P., and Adant, M., *Le Sang,* 1954, v25, 193.

11. Seigel, M., and Cliffton, E. E., *J. Gen. Physiol.,* 1957, v40, 377.

12. Basinger, C., and Allen, J. C., *J. Lab. and Clin. Med.,* 1951, v38, 784.

13. Page, E. W., Fulton, L. D., and Glendening, M. B., *Am. J. Obstet. and Gynec.,* 1951, v61, 1116.

14. Ware, A. G., Murphy, R. C., and Seegers, W. H., *Science,* 1947, v106, 618.

15. Murphy, R. C., and Seegers, W. H., *Am. J. Physiol.,* 1948, v154, 134.

16. Soulier, J. P., Alagille, D., and Larrieu, M. J., *Semaine Hôp. Paris,* 1956, v32, 1.

17. Vanderbroucke, J., and Verwilghen, R., *Le Sang,* 1955, v26, 699.

18. Alexander, B., Goldstein, R., Rich, L., Le Bolloc'h, A. G., Diamond, L. K., and Borges, W., *Blood,* 1954, v9, 843.

19. Penick, G. D., *Fed. Proc.,* 1957, v16, 369.

20. Macfarlane, R. G., *Physiol. Rev.,* 1956, v36, 479.

Received May 6, 1957. P.S.E.B.M., 1957, v96.

Pathogenesis of Arteriolar Necrosis of Malignant Hypertension.* (23497)

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Fibrinoid degeneration and necrosis of the walls of small arteries and arterioles, typical of the malignant phase of hypertension, have been observed in various tissues and organs of dogs with experimental renal hypertension which is accompanied by impairment of renal excretory function(1,2). The necrotizing arteriolar lesions occur in the rat also, when impairment of renal excretory function accompanies experimental renal hypertension(3). Various views concerning the pathogenesis of the necrotizing lesion, based mainly upon studies carried out on the rat, have been published, and these have been the subject of recent reviews(3,4). One concept of the pathogenesis of arteriolar necrosis which has won wide acceptance is that it is due solely to elevated blood pressure(5-9). Byrom and Dodson(7), have even asserted that sudden, intermittent increases of intra-arterial pressure, induced for only a short period, in normal rats, by a series of rapidly repeated, sudden, forcible, intracarotid injections of warm Ringer's solution, resulted in the production, in a few days, of what they interpreted as arterial and arteriolar necrosis, in the kidneys. It did not occur in kidneys the vascular bed of which had been protected from the sudden rise in intravascular pressure by occlusion of the main renal artery just prior to and during the injections. They expressed the view that, although the necrotizing arteriolar lesions are the result of elevated blood pressure alone, yet, once produced, they might lead to a vicious circle whereby the damaged kidney could perpetuate the existing hypertension. Although these results of Byrom and Dodson (7) have not been confirmed(4), nevertheless this concept has been invoked to explain the pathogenesis of malignant hypertension. It seemed of importance, therefore, to repeat their experiments and, in order to make the test more rigid, it was decided to attempt to produce even greater sudden increases of arterial pressure than were attained by them. The maximum rise reported by Byrom and

* This investigation was supported by grants from U. S. Public Health Service.

Dodson† was 90 mm Hg.

Method. In this study, young, female, albino rats varying from 170 to 220 g were used. The rises of blood pressure in the lower abdominal aorta caused by a series of sudden injections of Ringer's solution through needles of various gauges from #22 to #19 were first determined in a number of rats anesthetized with ether which were sacrificed immediately after completion of the injections. In these rats the left carotid artery was first cannulated with a blunt hypodermic needle and the abdominal aorta was cannulated just below the origin of the main renal arteries with a glass cannula measuring 0.8 mm in internal diameter at the tip. The cannula in the aorta was connected by plastic tubing with a mercury manometer (1.5 mm bore) and the animal was then heparinized. The intracarotid needle was connected by means of a three-way stopcock to a 2 cc insulin-type syringe and to a bottle of sterile Ringer's solution kept at 40°C. This arrangement permitted the filling of the syringe for rapidly repeated injections. The injections were made by hand, with as great force as possible, and the consequent rises of pressure in the lower abdominal aorta at the level of the origin of the renal arteries was observed by another person. It was found that the sudden, forcible injection of 2 cc of saline through needles of increasing gauge, gave increasing maximum elevations of blood pressure, but the maximum recorded with any needle smaller than a #19 gauge was 86 mm, whereas with a 19 gauge needle, the maximum attained in any animal was always well above 100 mm Hg above the basal level. In 8 rats in which a 19 gauge needle was used and the rises in pressure were determined, the maximum rises varied from 138 to 194 mm Hg. In all the rats the rise in pressure was immediate and it began to drop even before the injection was completed. The injection of larger amounts of fluid (5 and 10 cc) caused smaller rises. It was decided, therefore, to use the 19 gauge needle and 2 cc of Ringer's for this experiment.

† Determined in only one rat. Personal communication.

Experiments. For the definitive experiment, which required the survival of the animals for four days, the procedure described above was followed, except that the aorta was not cannulated for the determination of the blood pressure, and the blood was not heparinized. Thirty female albino rats were used, and every animal received 15 intracarotid injections of 2 cc of sterile Ringer's solution, through a #19 gauge needle, with an interval of 1 minute between injections. Upon completion of the injections, the needle was withdrawn from the carotid artery, the vessel was ligated, and the incision in the neck was closed by means of interrupted stitches. The animals recovered quickly from the operation and were sacrificed with ether at the end of the fourth day after the injections.

Results. At autopsy, in the gross, there were no significant changes in any of the organs. Pieces of tissue from the heart, stomach, duodenum, pancreas, mesentery, kidneys and, in some instances, brain, liver and adrenals were fixed in 10% formalin in normal saline, sectioned and stained with hematoxylin and eosin, for microscopic examination. More sections of the kidneys were made than of any other organ or tissue, because this was the main organ studied by Byrom and Dodson. Microscopic examination of these sections showed that in none of the 30 rats was there a single instance of fibrinoid degeneration, necrosis or inflammation of large or small arteries or arterioles in any organ or tissue, and even periarteritis nodosa did not occur in any of these animals. The findings were essentially negative.

Discussion. The results of this experiment make no positive contribution to the elucidation of the pathogenesis of arteriolar necrosis. We believe, however, that they tend to negate one of the most widely accepted views of the pathogenesis of fibrinoid necrosis of arterioles, namely, the mechanical effect of elevated blood pressure alone. In experiments similar to the present one, Johnston and Marples(4) also failed to observe necrotizing arterial and arteriolar lesions, but their failure to obtain them can be attributed to inadequate rises of blood pressure produced because they used a

#21 gauge needle. Similarly, the vascular lesions observed in a few animals by Masson and collaborators(10) certainly cannot be attributed to great increases in intravascular pressure (their recorded maximum is about 60 mm Hg), because they used a #22 gauge needle. By the use of a 19 gauge needle, however, although we succeeded in producing great rises of pressure, yet we failed to find any degenerated or necrotic arterioles in any of our 30 rats. Fortunately, also, in not a single rat did periarteritis nodosa occur to complicate the result and confuse the issue. Byrom and Dodson(7) claimed to have observed what they called renal arteriolar necrosis in 10 out of 23 rats. Their illustrations, however, suggest that they were dealing with periarteritis nodosa, and even these vascular lesions were few in number. The illustrations of Masson *et al.*(10) are also of periarteritis nodosa. The question of the identity of periarteritis nodosa and the necrotizing vascular lesion of malignant hypertension has been a source of confusion and this will not be discussed further here because we failed to observe any such lesions in our rats. If it should be established eventually that the two conditions are identical, then the significance of the necrotizing vascular lesion, at least insofar as hypertension in the rat is concerned, will greatly diminish, because in some strains of rat periarteritis nodosa occurs even in the absence of hypertension(3,11), or of impairment of renal excretory function. It is probable, however, that they are not identical, and it is therefore equally probable that the experimental conditions, as outlined by Byrom and Dodson, had nothing to do with the development of the vascular lesions which they observed in their rats.

Summary. Repeated intracarotid injections of warm, sterile, Ringer's solution, carried out under high pressure in control rats, caused transient rises of intra-aortic (lower abdominal) blood pressure, up to 194 mm Hg above the basal level. Thirty rats thus treated failed to show the development of arterial or arteriolar lesions of any kind, in the kidney or any other organ or tissue, when they were sacrificed at the end of 4 days after the completion of the injections. The results of this study fail to confirm the finding of Byrom and Dodson of necrotizing arterial and arteriolar lesions in the kidney as a result of repeated sudden increases of systemic intra-arterial tension, and do not lend support to their contention that elevated blood pressure alone is a sufficient condition for the production of the arteriolar lesions characteristic of malignant hypertension.

1. Goldblatt, H., *J. Exp. Med.,* 1938, v67, 809.

2. Goldblatt, H., and Kahn, J. R., *Blood, Heart and Circulation,* 1940, v13, 266.

3. Bali, T., and Goldblatt, H., *Exp. Med. and Surg.,* 1954, v12, 460.

4. Johnston, D. G., and Marples, G. A., *Angiology,* 1956, v7, 279.

5. Wilson, C., and Pickering, G. W., *Clin. Sc.,* 1938, v3, 343.

6. Wilson, C., and Byrom, F. B., *Quart. J. Med,* 1941, v10, 65.

7. Byrom, F. B., and Dodson, L. F., *J. Path. and Bact.,* 1948, v60, 357.

8. ———, *Clin. Sc.,* 1949, v8, 1.

9. Pickering, G. W., *Circulation,* 1952, v6, 599.

10. Masson, G. M. C., Corcoran, A. C., and Page, I. H., *Rév. Canad. de Biol.,* 1951, v10, 309.

11. Wilens, S. L., and Sproul, E. E., *Am. J. Path.,* 1938, v14, 201.

Received June 4, 1957. P.S.E.B.M., 1957, v96.

Effect of Gram-Negative Endotoxin on Nephrotoxic Serum Nephrosis in Rats.* (23498)

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Experimental renal disease produced in rats by intravenous administration of heterologous nephrotoxic antiserum has been studied by numerous investigators(1-7). The pathologic and clinical manifestations of the disease are variable, and depend upon such factors as manner of preparation and potency of the nephrotoxic serum(8), dose of serum given (3,9), time sequence of injections(7), and age and sex of the animals used(10). Recent efforts to enhance the nephrotoxicity of the serum with streptococcal culture filtrates were largely unsuccessful(11). Piel *et al.*(4) described the occurrence of bilateral renal cortical necrosis in rats given large amounts of nephrotoxic serum. This lesion was associated with deposits of hyaline fibrinoid material in the glomerular capillaries and was strikingly similar to the renal lesion of the generalized Shwartzman phenomenon in rabbits. Since the Shwartzman reaction had not been produced previously in rats, the lesion was considered to be non-specific and was not investigated further by these workers. Recently Gronvall and Brunson(12) reported that the generalized Shwartzman phenomenon could be produced regularly in rats by administration of Gram-negative endotoxin in conjunction with the high molecular weight acidic polymer, Liquoid (sodium polyanethol-sulfonate).

These observations suggested that Gram-negative endotoxin might potentiate the renal disease produced by administration of nephrotoxic serum and led us to investigate the effects produced in rats by intravenous injection of endotoxin in combination with nephrotoxic serum.

Material and Methods. Nephrotoxic serum was produced in rabbits by repeated intraperitoneal injection of rat kidney homogenate prepared according to the method described by Heymann and Lund(3). Lots of nephrotoxic serum from several rabbits were pooled and the potency of the material standardized. Sprague-Dawley rats of both sexes weighing 250-350 g were utilized as a source of renal tissue for preparation of kidney homogenate. The potency of the nephrotoxic serum was established by noting the dosage required to produce a severe nephrotic syndrome in rats. 158 female rats of the Sprague-Dawley strain, weighing 100 to 175 g were used in the study. These were fed Purina Fox Chow and had free access to water. All injectable solutions were made up to a volume of 1 cc. Animals given 2 substances thus received 2 cc of solution. All injections were given intravenously by tail vein. The numbers of animals used in the various experiments are tabulated. Animals dying during the experiment were autopsied and blocks of heart, lungs, spleen, liver and kidney were placed in 10% neutral formalin and sectioned in the usual manner for microscopic examination. *E. coli* endotoxin (lipopolysaccharide Rx B35527) was generously supplied by Mr. Aaron Lane of the Difco Laboratories, Detroit, Mich. Twenty-four hour urine specimens were collected in metabolic cages from rats which had free access to water only. Urinary proteins were measured early in the disease and again at the end of a week or 10 days using the Biuret method with a Bausch and Lomb Spectronic 20 colorimeter. All surviving rats were killed approximately 10 days after injection and autopsied as indicated above.

Results. Table I shows the survival rate and mean urinary protein excretion of rats

* Aided by grants from U.S.P.H.S., Am. Heart Assn., Minn. Heart Assn., Hill Family Foundation and Graduate School Research Fund of University of Minnesota.

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TABLE I. Effect of Nephrotoxic Serum on Survival of Rats Given Gram-Negative Bacterial Endotoxin.

Group	No. of rats	No. surviving	Protein excretion, mg/hr*
Normal rats	79	79	1.32
Normal rabbit serum (1 cc)	5	5	1.44
Endotoxin (250 γ)†	5	5	1.09
" (10 γ)	5	5	1.05
Normal rabbit serum (1 cc) + endotoxin (10 γ)	5	5	1.04
Normal rabbit serum (1 cc) + endotoxin (250 γ)	5	5	1.43
N.T.S.‡ (1 cc)	13	11	8.90
" (2 cc)	5	5	8.95
" (1 cc) + endotoxin (300 γ)	5	0	
" " + " (250)	8	3	13.5
Idem (100)	3	0	
" (50)	8	0	
" (10)	8	0	
" (5)	5	0	
" (1.0)	8	2	12.8
" (.5)	5	2	
" (.1)	5	5	9.47

* Mean mg/hr. † Lipopolysaccharide endotoxin from *E. coli*. ‡ Nephrotoxic serum.

given normal or nephrotoxic rabbit serum and *E. coli* endotoxin separately or in combination. All animals that were given normal rabbit serum or lipopolysaccharide endotoxin alone or in combination survived. Rats given nephrotoxic serum in doses (1-2 cc per rat) sufficient to produce proteinuria regularly survived 10 days. In contrast, when 1 cc of nephrotoxic serum was given together with endotoxin, a majority of the animals died in periods of 3 to 24 hours following injection. For example, in the groups given amounts of endotoxin varying from 300 γ to 5 γ, only 3 survived longer than 24 hours. In each instance death was preceded by prostration, lethargy, ruffling of the fur, bloody nasal discharge and diarrhea. The survival rate increased when endotoxin was given in doses of less than 1 γ. At 0.5 γ 2 of 5 rats survived, and all animals given 1 cc of nephrotoxic serum in combination with 0.1 γ *E. coli* endotoxin survived.

Urinary protein excretion of rats given nephrotoxic serum alone in the dosages used was about 7 times that of normal rats. The protein excretion of animals injected with normal rabbit serum or endotoxin alone and in combination varied little from the normal controls. Proteinuria among the few animals which survived injections of nephrotoxic serum and endotoxin was of approximately the same magnitude as in rats given nephrotoxic serum alone.

Morphologic changes. Gross changes in the animals which died following combined nephrotoxic serum and *E. coli* endotoxin injection included ascites, pulmonary hemorrhages and enlarged bluish-red kidneys with tense capsules. On cross section the kidney lesions were suggestive of early bilateral cortical necrosis and if the animals had survived longer it is probably that the characteristic changes of the generalized Shwartzman phenomenon would have been more apparent.

Microscopic examination of the kidneys from rats dying after simultaneous injection of endotoxin (all dosages) plus 1 cc of nephrotoxic serum revealed deposition of hyaline fibrinoid material in the glomerular capillaries together with tubular necrosis, eosinophilic staining tubular casts and proliferation of glomerular capillary endothelial cells (Fig. 1).

Kidneys from rats given nephrotoxic serum alone showed only a proliferation of endothelial cells similar to that which occurs in acute glomerulonephritis in man (Fig. 2).

Discussion. The relative resistance of rats to the lethal action of large doses of lipopolysaccharide endotoxin observed by others(13) is supported by our investigations. Intravenous injection of as much as 250 γ failed to kill

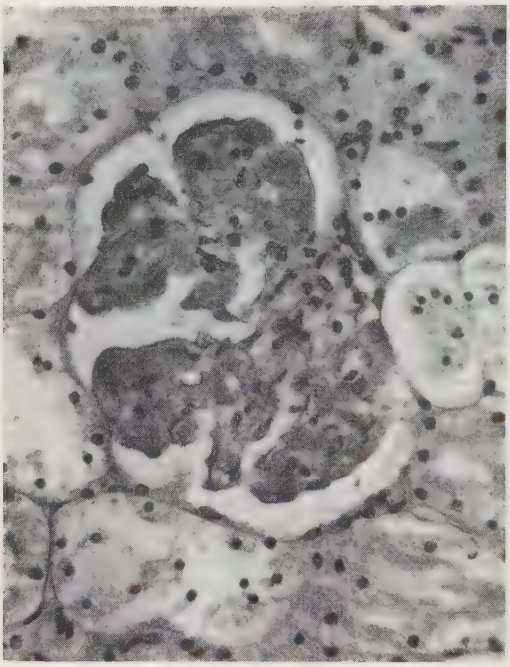

FIG. 1. Periodic acid-Schiff stain of a glomerulus from a rat given 1 cc of nephrotoxic serum simultaneously with 150 γ of *E. coli* endotoxin. Death occurred within 24 hours. Note moderate proliferation of capillary endothelial cells, glomerular capillary occlusion with Schiff-positive fibrinoid material, and early tubular necrosis. × 360.

any animals. The relatively high properdin values(14) found in this species might partially explain the rat's resistance to the lethal and necrotizing effects of endotoxin. One might also postulate a more rapid clearance of endotoxin by the reticuloendothelial tissues (15) in rats than in other animals. Whatever its basis, this resistance to endotoxin is readily overcome by a simultaneous injection of nephrotoxic serum. When given together with nephrotoxic serum, even minute amounts of endotoxin (0.5 γ) produced early death and the morphological picture of the generalized Shwartzman reaction. One hundred per cent survival of rats given 0.1 γ of endotoxin in combination with nephrotoxic serum indicates that a minimum amount of endotoxin necessary for the lethal effect has been reasonably well defined.

The mechanism by which nephrotoxic serum and Gram-negative endotoxin act together to produce this pronounced lethal effect in rats is unknown. However, the observations presented here are similar to those made previously in these laboratories(16-18) demonstrating enhancement of lethal and necrotizing effects of endotoxin in rabbits by prior treatment with cortisone and prior or simultaneous injection of thorotrast, trypan blue, colloidal iron or colloidal carbon. Thus, nephrotoxic serum might interfere with the capacity of the R. E. system to clear endotoxin from the blood. At present, we have no evidence to support this concept but experiments to test the possibility seem indicated.

Gronvall and Brunson(12) produced the generalized Shwartzman reaction and death in rats by administering endotoxin together with or prior to injection of sodium polyanethol sulfonate (Liquoid). It seems unlikely from the timing and requisite sequence of the injections in their studies that the polysaccharide polymer exerts its effect by producing "reticuloendothelial blockade." Investigations by Thomas, *et al.*(19) have indicated that other mechanisms are involved in the apparent synergism of large molecular weight acidic polymers and endotoxin. Experiments to test the possibility of these mechanisms being the basis for the synergistic action of nephrotoxic serum and Gram-negative endotoxin are in progress. These include variations in the sequence and time relationships of injections and attempts to prevent the renal lesions with heparin and nitrogen mustard(20,21). Preliminary studies(22) have shown a depletion of the heparin-precipitable protein nor-

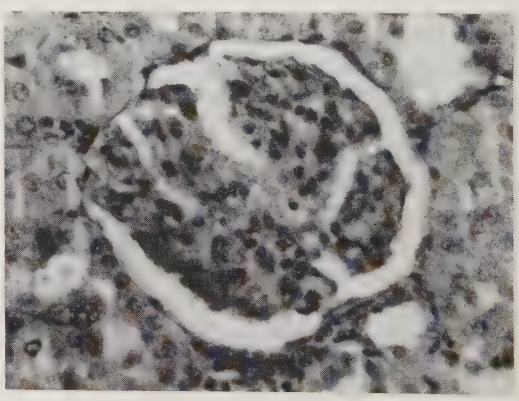

FIG. 2. Periodic acid-Schiff stain of a glomerulus from a rat given 1 cc of nephrotoxic serum alone. Killed 10 days after injection. Note proliferation of glomerular capillary endothelial cells and absence of Schiff-positive material. × 275.

mally present in rat plasma following injection of nephrotoxic serum alone or in combination with endotoxin. This observation suggests that nephrotoxic serum may have a similar action to the large molecular weight acidic polymers.

Our pathological studies indicate that still a third mechanism might be found basic to the synergistic action of nephrotoxic serum and Gram-negative endotoxin. Nephrotoxic serum produces proliferation and swelling of the endothelium of glomerular capillaries which might render these blood vessels more susceptible than normal to the necrotizing action of endotoxin. Similar changes produced in the general vascular system, even though not recognizable by light microscopy, might account for the lethal effect and the general vascular susceptibility to the necrotizing process.

These investigations may provide an explanation for the recent observations of Baxter and Goodman(7) who found that nephrotoxic serum incubated with rat intestine resulted in a product which killed all rats when injected intravenously. In contradistinction, nephrotoxic serum incubated with other organs and tissues had no such effect. It is likely that the lethal effect they observed was a function of a synergistic action of nephrotoxic serum and minute amounts of lipopolysaccharide endotoxin of bacterial origin derived from intestinal tissues. Likewise, the occasional instance of bilateral cortical necrosis observed by Piel *et al.*(4) in animals given nephrotoxic serum alone may have been due to a synergistic action of nephrotoxic serum and minute quantities of bacterial endotoxin absorbed from the bowel or endotoxin-like polysaccharides derived from other tissues.

More important are the implications of these experiments that a complicating factor otherwise of so little consequence to the rats can result in death and widespread fibrinoid necrosis when the kidneys have been damaged by nephrotoxic serum. Several diffuse renal diseases in man have a common pathological lesion in the end stage. One might speculate that the underlying disease process induces an extreme vulnerability of the vessels to the necrotizing actions of certain lipopolysaccharides. Thus, a "complicating factor" actually might induce the progressive renal disease and could be primarily responsible for the morphological characteristics of the end state. Stetson (23) and Schwab *et al.*(24) found that group A streptococci contain an endotoxin similar to Gram-negative bacterial endotoxins. Landy (25) discovered polysaccharides with endotoxin-like action to be widely distributed in nature, including mammalian tissues. These observations permit postulation that toxins capable of producing the pathological lesions described here might be derived from sources other than Gram-negative bacteria. Further studies to evaluate this hypothesis are in progress.

Summary. 1) Intravenous injection of small amounts of nephrotoxic serum produced a syndrome in rats whose characteristics include proteinuria and proliferation of glomerular endothelial cells. This dosage of nephrotoxic serum was well tolerated by the rats. 2) Rats were shown to tolerate *E. coli* lipopolysaccharide endotoxin over a wide range of dosages. 3) Simultaneous injection of nephrotoxic serum and minute amounts of Gram-negative bacterial endotoxin resulted in acute death in a high percentage of rats and renal lesions resembling those of the generalized Shwartzman reaction. 4) The possible implications of these observations on susceptibility to necrotizing effects of endotoxin-like substances and on the pathogenesis of renal disease are discussed.

1. Smadel, J. E., *J. Exp. Med.*, 1936, v64, 941.

2. ———, *idem*, 1937, v65, 541.

3. Heymann, W., and Lund, H. Z., *Pediatrics*, 1951, v7, 691.

4. Piel, C. F., Dong, L., Modern, F. W. S., Goodman, J. R., and Moore, R., *J. Exp. Med.*, 1955, v102, 573.

5. Ehrich, W. E., Forman, C. W., and Seifter, J., *Arch. Path.*, 1952, v30, 311.

6. Solomon, D. H., Gardella, J. W., Fanger, H., and Ferreber, J. W., *J. Exp. Med.*, 1949, v90, 267.

7. Baxter, J. H., and Goodman, H. C., *ibid.*, 1956, v104, 487.

8. Ehrich, W. E., Forman, C. W., and Seifter, J., *Arch. Path.*, 1952, v54, 463.

9. Luetscher, J. A., Piel, C. F., and Curtis, R. H., *J. Chronic Dis.*, 1955, v1, 442.

10. Lippman, R. W., Marti, H. V., Jacobs, E. E., and Campbell, D. H., *Arch. Path.,* 1954, v57, 254.

11. Wakim, K. G., and McKenzie, B. F., *Fed. Proc.,* 1957, v16, 132.

12. Gronvall, J. A., and Brunson, J. G., *Arch. Path.,* 1956, v62, 324.

13. Shwartzman, G., *Phenomenon of Local Tissue Reactivity,* New York, Paul B. Hoeber, 1937, p140.

14. Pillemer, L., Blum, L., Lepow, I. H., Ross, O. A., Todd, E. W., and Wardlaw, A. C., *Science,* 1954, v120, 279.

15. Beeson, P. B., *J. Exp. Med.,* 1947, v86, 29.

16. Thomas, L., and Good, R. A., *ibid.,* 1952, v95, 409.

17. Good, R. A., and Thomas, L., *ibid.,* 1952, v96, 625.

18. Smith, R. T., Thomas, L., and Good, R. A., PROC. SOC. EXP. BIOL. AND MED., 1953, v82, 712.

19. Thomas, L., Smith, R. T., and Von Korff, R. W., *J. Exp. Med.,* 1955, v102, 263.

20. Stetson, C. A., and Good, R. A., *ibid.,* 1951, v93, 49.

21. Thomas, L., and Good, R. A., *ibid.,* 1952, v96, 605.

22. The authors, unpublished data.

23. Stetson, C. A., *J. Exp. Med.,* 1956, v104, 921.

24. Schwab, J. H., Watson, D. W., and Cromartie, W. J., PROC. SOC. EXP. BIOL. AND MED., 1953, v82, 754.

25. Landy, M., and Shear, M. J., *J. Exp. Med.,* 1957, v106, 77.

Received June 19, 1957. P.S.E.B.M., 1957, v96.

Blood Pressure of the Normal Rhesus Monkey. (23499)

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This laboratory has been interested in developing procedures for evoking hypertension in subhuman primates such as the rhesus monkey(1). There being essentially no definitive data on variations in blood pressure of the normal rhesus monkey, determination of the limits of such variations was a necessary preliminary to studies on hypertension. This report includes observations on blood pressure variations in normal and splenectomized monkeys acquired with 2 indirect methods as well as comparisons of these indirect readings with measurements of the arterial pressure obtained directly with a strain gauge.

Materials and methods. Animals. Young male and female rhesus monkeys weighing from 3 to 12 kilos were used. Details of the care and feeding of animals in this colony have been described by Schmidt and Genther (2). Three groups of monkeys were employed in these studies. The first group comprised 14 normal monkeys on which blood pressure measurements were made repeatedly over a 2-month period using the 2 indirect blood pressure methods described below. The second group consisted of 27 splenectomized animals on which control blood pressures were measured for 2 months in the same fashion as the first group. These monkeys had been infected with malaria, their infections cured by various chemotherapeutic agents, and this fact confirmed by splenectomy. The blood pressure studies were begun 2 or more months after splenectomy. After the control period the majority of these animals were used in studies on experimental hypertension. However, 6 were observed as controls for an additional 12 months, observations on these animals providing data on stability of simian blood pressure levels over an extended period. The third group consisting of 46 anesthetized monkeys was used in comparing the direct and indirect blood pressure methods. Of these, 43 had no known cardiovascular or renal disease while the remaining 3 had moderate experimental hypertension. The procedure used in handling the monkeys was an important factor in securing reproducible blood pressure measurements. The monkeys were housed in large cages, each containing 6 to 10 animals. The cages opened individually onto a narrow corridor to which animals were admitted one at a time. The monkey was caught on its first run, then allowed to rest

with minimal manual restraint for 5 minutes prior to beginning blood pressure measurements. During the measurements the animal was held in a sitting position with its legs pinned gently over the edge of the table by the thigh of the assistant. The monkey's head was held in the assistant's right hand and its left arm (if this extremity was to be used for pressure measurement) extended horizontally by the assistant's left hand. Most monkeys could be trained to sit quietly; however, those that showed no evidence of training after a 2- to 3-week period were returned to stock.

Blood pressure measurements. Indirect methods. Indirect measurements of systolic and diastolic blood pressures were made essentially as in man using either a "cloth" or "metal" cuff. The cloth cuff, a modified "newborn" cuff, consisted of an inflatable rubber bag, 1 by 2 inches, enclosed in a cloth cover 1¼ by 14 inches. Ties sewed to the end of the cover facilitated rapid application to the animal's arm. The cuff was wrapped snugly around the extended left (or right) arm as close to the body as possible. Care was taken not to occlude venous return; when the cuff was applied loosely, high and variable readings resulted. The "metal" cuff consisted of a short metal or plastic cylinder fitted inside with an airtight rubber sleeve. Air pumped into the space between the cylinder and sleeve through a tubulature forced the rubber sleeve against the arm, compressing the arm equally from all directions. Since the diameter of the arm varies with the size of the monkey, a series of cuffs with internal diameters of 45, 55, 65 and 75 mm and varying in length from 45 to 65 mm were employed. These sizes were adequate for monkeys weighing from 2 to 12 kilos. The Korotkoff pulse sounds were rather faint in some animals; therefore, to obtain the best sound it was important to use a small stethoscope bell (15 mm I.D.) and to place the bell directly over the artery. Pressure readings were made in triplicate, the average to the nearest 5 mm being recorded. In quiet, trained monkeys these readings never varied by more than 10 mm Hg; wider variations indicated that the animal was excited or insufficiently rested. Since excited animals usually had elevated cardiac and respiratory rates as well, these latter measurements, which were obtained routinely, provided additional checks on the reliability of the blood pressure readings.

Direct measurements. For these measurements the brachial or femoral artery was exposed under pentobarbital anesthesia and cannulated with a short, blunt 18- or 19-gauge needle. The needle was connected to a transducer (Model P23A Statham strain gauge), and pressure changes were recorded photographically (Cambridge Simplitrol electrocardiograph). The indirect blood pressure readings were compared with direct blood pressure measurements in 46 monkeys, a total of 505 comparisons being made of the direct pressure in the brachial or femoral artery with the indirect pressure in the arm measured at the same time with a cloth or metal cuff. The indirect pressure measurements were never biased by knowledge of the direct readings being recorded simultaneously since the direct readings were calculated from photographic records after an experiment was concluded.

Results. Blood pressure range in control monkeys. Mean systolic and diastolic blood pressures of 14 control monkeys determined indirectly with the cloth and metal cuffs are presented in Table I. It is evident that the cloth cuff readings were substantially higher than those obtained with the metal cuff, the cloth cuff technic giving an average systolic and diastolic pressure for the group of 159/127 mm Hg as contrasted with group average of 118/90 mm for the metal cuff. Mean blood pressures in different animals varied widely; with the cloth cuff the mean values for individual monkeys ranged from 137/112 to 188/152, with the metal cuff from 103/78 to 132/101 mm.

Data on splenectomized monkeys, also summarized in Table I, show that mean pressures in this group were slightly but not significantly lower than those of the intact control group. Thus the splenectomized group had average systolic and diastolic pressures of 149/116 and 112/84 mm Hg for the cloth and metal cuffs respectively and exhibited the

TABLE I. Arterial Blood Pressures of Control and Splenectomized Monkeys Measured by Two Indirect Methods.

Type of monkey	No. of Monkeys	No. of Observations	Cuff	Blood pressure in mm Hg Systolic Mean ± S.D. (range)	Blood pressure in mm Hg Diastolic Mean ± S.D. (range)
Control	14	315	Cloth	158.8 ± 12.8 (137–188) (10–17)	126.7 ± 11.9 (112–152) (8–15)
	14	315	Metal	117.5 ± 8.1 (103–132) (6–10)	89.5 ± 7.4 (78–101) (5–10)
Splenectomized	27	526	Cloth	148.9 ± 11.9 (123–170) (8–16)	115.9 ± 10.8 (94–134) (7–16)
	27	526	Metal	111.5 ± 7.1 (94–131) (4–12)	83.2 ± 7.1 (70–101) (5–10)

same wide range of mean values in individual monkeys as was observed in the control group. The standard deviations of the metal cuff readings were always smaller than those of the corresponding cloth cuff measurements, *i.e.*, 7 and 12 mm respectively. Thus for an individual monkey systolic and diastolic pressures would vary within a 30 mm range when measured with the metal cuff and within a 50 mm range when the cloth cuff was used. Pressures were more variable in some monkeys than in others, but degree of variation was not related to blood pressure level. There was relatively good correlation between cloth and metal cuff readings in a given monkey; the correlation coefficient (r) of the 2 mean systolic readings was 0.92 and for the 2 mean diastolic pressures the coefficient was 0.85.

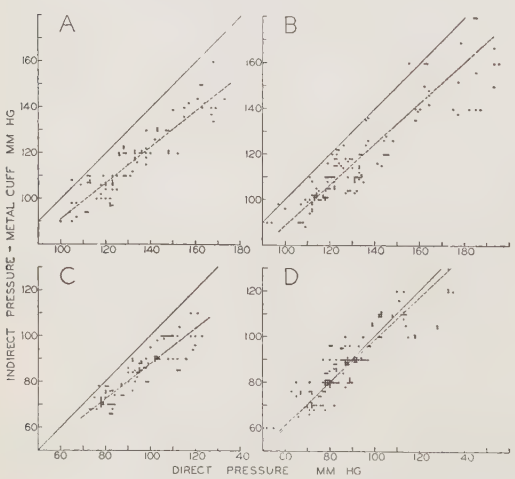

FIG. 1. Comparison of indirect (metal cuff) systolic pressure readings with systolic pressures measured directly in brachial (A) or femoral (B) artery. Indirect diastolic readings compared with direct diastolic pressures in brachial (C) or femoral (D) artery. Solid line is that of equal values on the 2 scales.

The relative stability of the blood pressure in non-treated monkeys is illustrated by data obtained on the 6 splenectomized but otherwise normal animals studied for 12 months following their initial 2 month observation period. At no time did mean systolic or diastolic pressures of any of these monkeys vary by as much as 10 mm Hg from the mean values obtained for the same animal during the initial observation period.

Comparison of direct and indirect blood pressures. The relationships between the 2 indirect blood pressure readings and the intra-arterial pressure measured directly with a strain gauge were defined by making 8 types of comparisons. The results are shown in Fig. 1 and 2. Each dot represents an associated pair of direct and indirect pressure readings obtained simultaneously in the same monkey. The heavy broken line in each figure represents the regression, calculated by the method of least squares, of the indirect pressure on the direct pressure. The thin solid line in each figure is the line of equal values. In Fig. 3 these 8 regression lines designated A to H have been superimposed to permit easier comparison.

Comparison of systolic and diastolic pressures measured directly in the brachial or femoral artery with the corresponding indirect readings in the arm measured with the metal cuff (Fig. 1; curves A and C, brachial; curves B and D, femoral) indicates that the direct readings were consistently higher than the indirect (metal cuff) readings except for curve

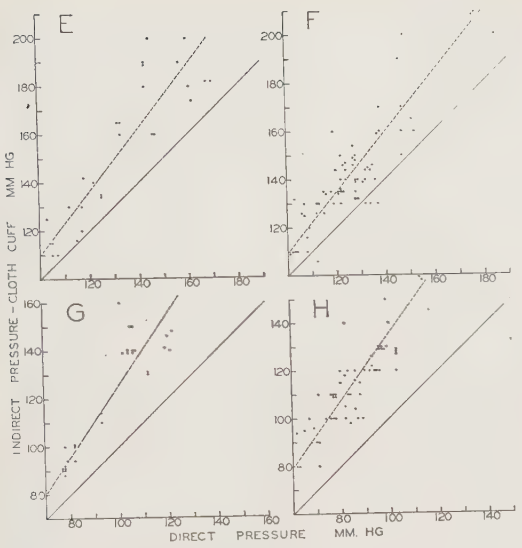

FIG. 2. Comparison of indirect (cloth cuff) systolic pressure readings with systolic pressures measured directly in brachial (E) or femoral (F) artery. Indirect diastolic readings compared with direct diastolic pressures in brachial (G) or femoral (H) artery. Solid line is that of equal values on the 2 scales.

D, in which the indirect diastolic pressure in the arm is compared to the direct diastolic pressure in the femoral artery. The direct readings exceeded the corresponding indirect readings by about 10 mm Hg in the normotensive range. The opposite relationship was found between direct pressure and pressure estimated indirectly using the cloth cuff (Fig. 2). In this case the direct measurements were always lower than the indirect (cloth cuff) readings. As with the metal cuff, the difference became more pronounced at higher blood pressure levels.

These data indicate several noteworthy trends. In the first place, cloth cuff readings were always higher and slightly more variable than the metal cuff readings, the difference between the 2 readings becoming slightly more pronounced at higher blood pressure levels. Secondly, direct blood pressure measurements obtained with a strain gauge fell about midway between the cloth and metal cuff readings. Thus the average blood pressure of normal monkeys, if it could be measured directly without anesthetizing or exciting the animals, would be about 130/100 mm Hg

Discussion. There are very few reports in the literature on blood pressure variations in normal or hypertensive monkeys. Attempts to use the indirect method of McGregor(3) employed by Goldblatt(4) gave extremely variable results even in anesthetized animals. Direct femoral puncture, the procedure employed by Frank and Wakerlin(5) also yielded unsatisfactory results. This method is technically difficult and gives only an undefined average pressure when a mercury manometer is used. Restraint of the monkey on a board, required by either procedure, often excites the animal, causing an abnormal elevation of the blood pressure.

The indirect procedures described in this report are applicable to either unanesthetized or anesthetized animals. The methods possess certain inherent sources of error characteristic of most indirect procedures(6,7). However, by careful attention to detail it has been possible to define accurately blood pressure levels in untreated animals and to establish the significance of blood pressure changes following several types of treatments (1). Roughly 11,000 determinations on about 90 animals have been made to date; in some

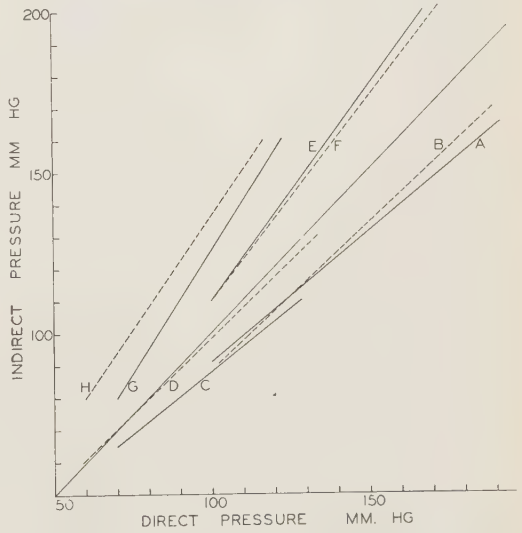

FIG. 3. The 8 regressions of indirect on direct pressure measurements shown in Figs. 1 and 2 are superimposed. A and B, metal cuff systolic; C and D, metal cuff diastolic; E and F, cloth cuff systolic; G and H, cloth cuff diastolic. In A, C, E and G direct pressure was measured in brachial artery and in B, D, F and H, in femoral artery. The thin solid line is that of equal values on the 2 scales.

animals blood pressure levels have been followed for as long as 2 years. The rather unexpected stability of systolic and diastolic blood pressures in rhesus monkeys makes this animal admirably suited for experiments in which blood pressure changes accompanying an experimental procedure are to be investigated.

Summary. Arterial blood pressures have been determined in unanesthetized monkeys by 2 indirect methods. Using a small "newborn" cuff (cloth cuff) on the upper arm and auscultation of the sounds in the brachial artery, mean blood pressure in 14 normal monkeys was found to range from 137/112 to 188/152 mm Hg with a group average of 159/127. With a cuff consisting of a rigid metal shell enclosing a rubber sleeve (metal cuff), mean pressures ranged from 103/78 to 132/101, the group average being 118/90 mm. In 27 splenectomized, but otherwise normal, monkeys the blood pressures were slightly but not significantly lower. Metal cuff pressure readings were found to be consistently less variable than corresponding cloth cuff values, the respective average standard deviations in normotensive animals being 7 and 12 mm. Blood pressures in untreated animals estimated by either indirect method were relatively stable; in no instance did mean systolic or diastolic pressure during a 12-month period vary by as much as 10 mm Hg from the mean values obtained during an initial 2-month observation period. Simultaneous comparisons of indirect (cloth or metal cuff) and direct (strain gauge) blood pressure measurements in 46 anesthetized animals indicated that the metal cuff readings were lower and the cloth cuff readings higher than measurements obtained directly in the same artery with a strain gauge. The blood pressure as measured directly with a strain gauge fell about midway between the corresponding cloth and metal cuff readings.

1. Smith, Carl C., and Ansevin, A., PROC. SOC. EXP. BIOL. AND MED., 1957, v96, 432.

2. Schmidt, L. H., and Genther, C. S., *J. Pharmacol. Exp. Therap.*, 1953, v107, 61.

3. McGregor, L., *Arch. Path.*, 1928, v5, 630.

4. Goldblatt, H., *J. Exp. Med.*, 1937, v65, 671.

5. Frank, M. H., and Wakerlin, G. E., *Fed. Proc.*, 1954, v13, 47.

6. Wilhelmj, C. M., Waldman, E. B., and McGuire, T. F., *Am. J. Physiol.*, 1951, v166, 296.

7. Van Bergen, F. H., Weatherhead, D. S., Treolar, A. E., Dobkin, A. B., and Buckley, J. J., *Circulation*, 1954, v10, 481.

Received June 27, 1957. P.S.E.B.M., 1957, v96.

Experimental Hypertension in the Rhesus Monkey. (23500)

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There have been few reported studies on induction of experimental hypertension in the rhesus monkey. The investigation described here deals with attempts to provoke hypertension in this species by procedures which have been successful in rats, dogs, and other experimental animals. These procedures included temporary unilateral renal artery occlusion(1), unilateral or bilateral silk perinephritis(2), unilateral or bilateral constriction of the renal arteries with silk ligatures (3) or clamps(4), and oral administration of excessive amounts of sodium chloride(5).

Materials and methods. Animals. Sixty rhesus monkeys (*Macaca mulatta*), 26 males and 34 females, weighing from 3 to 12 kilos were used in this study. General care and feeding followed the pattern described by Schmidt and Genther(6). All animals had been infected previously with malaria, their infections cured by various chemotherapeutic agents, and this fact proved by absence of parasitemia following splenectomy. These monkeys were indistinguishable from intact control animals insofar as range and variability of systolic and diastolic blood pressures

are concerned(7). *Producing hypertension. Temporary unilateral renal artery occlusion* (1). In 7 monkeys blood flow to the right kidney was interrupted for one hour; in 2, flow was interrupted for 2 hours. After the prescribed time interval the clamp occluding the renal artery was removed and contralateral nephrectomy was performed immediately thereafter. *Silk perinephritis*(2). A. In 6 monkeys the right kidney was exposed through a lumbar incision and wrapped snugly in silk cloth, care being taken not to obstruct blood flow through the pedicle. B. In 6 monkeys the right kidney was wrapped as in A and contralateral nephrectomy was performed immediately thereafter. C. In 8 monkeys both kidneys were wrapped in silk as in A, the interval between the 2 wrappings varying from 7 to 21 days. *Stenosis of renal arteries with ligatures*(3,8). In 16 monkeys the renal arteries were exposed retroperitoneally and a loop of heavy silk thread placed around both the artery and a stylet held adjacent to it. After the tie was made, the stylet was withdrawn. Of the 16 monkeys, 12 had been operated upon 5 months previously (renal artery occlusion or silk perinephritis) but had remained normotensive. *Bilateral renal artery constriction with clamps* (4). In 15 animals not used previously in this investigation both renal arteries were constricted with small adjustable clamps. These clamps were constructed from one-inch strips of 1/16 inch thick methyl methacrylate (Lucite), ¼ inch wide and bent in the shape of a flat "V", the arms of which could be closed by a stainless steel adjusting screw. In all instances the right renal artery was constricted first, the left renal artery 26 to 28 days later. Following the suggestions of Wakerlin(9), Schroeder and Goldman(10), and Goldblatt(4), attempts were made to reduce flow to one-third of normal. This point was difficult to judge even with transparent clamps. Seven of the animals developed uremia following application of the second clamp; in 5 of these, loosening or removing the second clamp failed to lower the uremia or to prolong life. *Sodium chloride administration*(5). The effect of excessive dosage of NaCl was studied in 29 monkeys. Twenty of the group had been operated upon 4 to 6 months previously. Three of these had been subjected to temporary unilateral renal artery occlusion; 5 to unilateral silk perinephritis; 4 to unilateral silk perinephritis and contralateral nephrectomy; and 8 to bilateral silk perinephritis. Three others had had one kidney removed several months previously, while the remaining 6 monkeys were untreated controls. For the first 16 days NaCl was administered orally as a 4% solution at a daily dose of 400 mg/kg. For the following 14 days this same dose was supplemented by adding NaCl in a concentration of 0.5% to the diet and drinking water. For the final 22-day period the oral dose was raised to 800 mg/kg and the concentration in the diet to 1%. The average daily salt intake was 400 mg/kg for first 16 days, 1300 to 1800 mg/kg for the second period, and 2100 to 2600 mg/kg for the final 22-day period. *Blood pressure measurements and incidental studies.* Systolic and diastolic blood pressures were measured indirectly by the "newborn" cloth cuff and/or metal cuff procedures described by Smith and Ansevin(7). On each animal measurements were made 2 or 3 times each week during a control period of 2 or more months and at similar intervals during the experimental period. All monkeys were under close daily observation and were weighed weekly. The concentrations of sodium, potassium, and urea nitrogen in serum were determined biweekly or more frequently when indicated. Necropsies were performed immediately after death on all animals that succumbed. All surviving animals were sacrificed. In all cases weights of the heart, adrenals, liver, and kidneys were obtained. Segments of these and other organs were taken for histopathologic examination. The results of these studies will be reported separately.

Criteria of hypertension. The variation in the blood pressure of the normal rhesus monkey has been dealt with in the previous paper (7). As illustrated by the data in Fig. 1A, blood pressures of untreated animals were remarkably stable. Over a 12-month observation period average systolic and diastolic

TABLE I. Effectiveness of Various Experimental Procedures in Inducing Hypertension.

Exp. procedure	No. of monkeys used in exp. procedure: Total	Blood pressure response: None	Hypertension: Slight	Moderate	Severe	No. dying in acute renal failure
Unilateral renal artery occlusion	9	5				4
Silk perinephritis						
A. Unilateral	6	5		1		
B. Unilateral, contralateral nephrectomy	6	6				1
C. Bilateral	8	6	1	1		
Renal artery stenosis with silk ligatures	16	0	1	2	2	11
Bilateral renal artery constriction with plastic clamps	15	3	1	1	3	7

blood pressures never varied more than ± 10 mm from the mean pressure levels obtained during the initial 2-month standardizing period. This stability in the blood pressure of the normal monkey made it comparatively easy to establish criteria for hypertensive response. Monkeys whose blood pressures became consistently elevated above their pretreatment levels were considered to be hypertensive, the degree of elevation being classified as slight, moderate or severe. Elevations in mean pressure of 15 to 30 mm as measured with the metal cuff were classified as slight, elevations of 30 to 50 mm as moderate, and elevations greater than 50 mm as severe hypertension.

Results. Data in Table I show that restriction of the total blood supply to the kidney was by far the most effective method of inducing hypertension. Thus all 5 monkeys which survived constriction of the renal arteries through use of silk ligatures developed some degree of hypertension. In 2 the reaction was severe (Fig. 1D), in 2 moderate and in one slight. When constriction was achieved with plastic clamps, 5 of 8 surviving animals exhibited hypertensive changes, 3 being classified as severe and one each as moderate (Fig. 1B) or slight.

The hypertensive changes in the above animals were obtained at the price of a high overall mortality. Thus 18 of the 31 animals in the combined silk ligature-plastic clamp groups died in acute renal failure 3 to 23 days after the final operation. Two other animals with hypertensive responses also succumbed. One animal developed severe hypertension 2 weeks after the second clamp had been applied and died 7 weeks later in renal failure (Fig. 1C). The remaining monkey presented an unusual blood pressure response which will be discussed subsequently.

Attempts to produce hypertensive changes by other procedures were relatively non-productive. Temporary unilateral renal artery occlusion produced no elevation in blood pressure. None of the animals subjected to silk perinephritis, either unilaterally or bilaterally, developed severe hypertension. One animal with one kidney wrapped in silk developed moderate hypertension. Necropsy 4 months after operation revealed gross suppuration in the area of the silk. Of the 8 animals with bilateral silk perinephritis, one developed moderate and another slight hypertension during an observation period of approximately 11 months.

In the monkeys fed excessive amounts of sodium chloride in their diet and drinking water, blood pressure responses were variable. Sustained elevations in pressure occurred in only 3 animals: one monkey with temporary unilateral renal occlusion; one unilaterally nephrectomized animal; and one control monkey. The experiment was terminated after 52 days when many of the monkeys became weak, began to lose weight and appetite, and showed evidence of enteric irritation.

Discussion. There have been few reports on production of experimental hypertension in the monkey although this condition has been studied extensively in other laboratory ani-

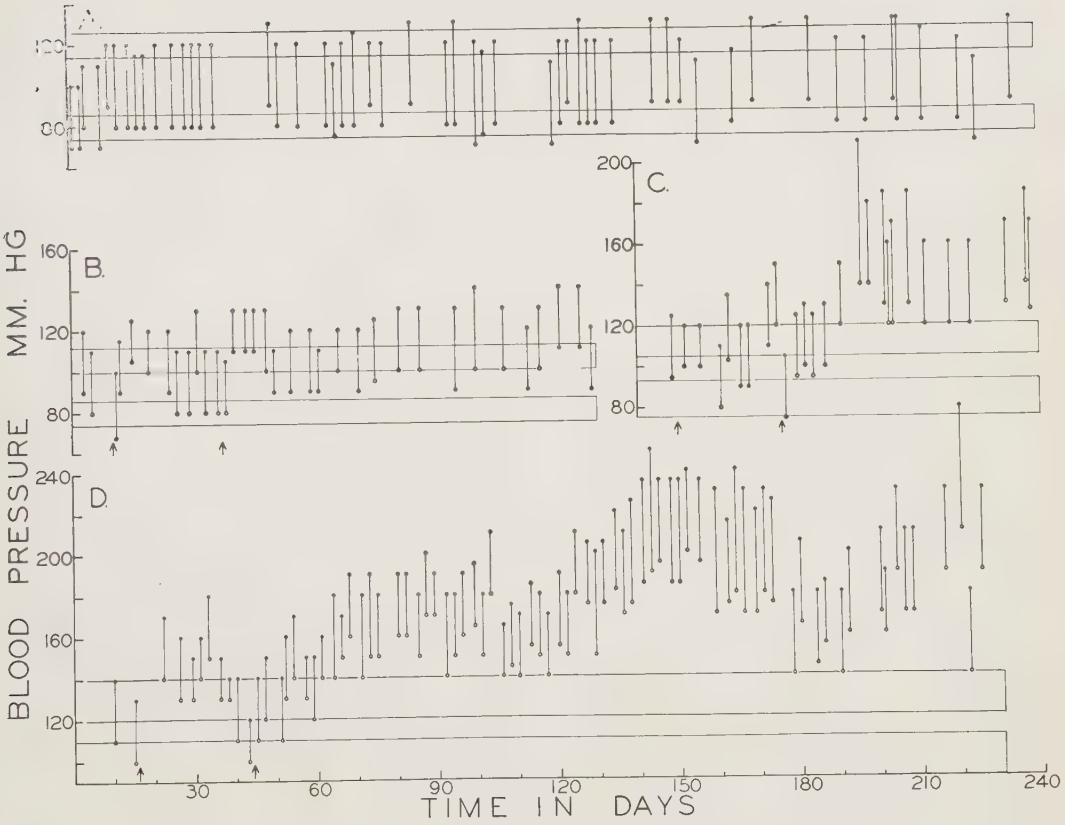

FIG. 1. Blood pressure readings in a control monkey and in monkeys with experimental hypertension; systolic (●) and diastolic (○). In each graph the upper box represents mean preoperative systolic pressure ± one standard deviation, the lower box, mean diastolic pressure ± one st. dev. Operative procedures are indicated by arrows. A. Control monkey; metal cuff reading. B. Slight to moderate hypertension following bilateral renal artery constriction with plastic clamps; metal cuff readings. C. Severe hypertension following bilateral renal artery constriction with plastic clamps. Monkey died in renal failure; metal cuff readings. D. Severe hypertension following bilateral renal artery stenosis with silk ligatures; cloth cuff readings.

mals. Goldblatt(4) described elevations in blood pressure in 3 of 5 monkeys following application of clamps to both renal arteries. In one animal indirect blood pressure measurements as high as 300 mm Hg were recorded; however, none of the animals had "malignant hypertension"(11). Frank and Wakerlin(12) dealt briefly with production of moderate hypertension in a larger series of monkeys using the Goldblatt method.

Of the 6 operative procedures compared in this study, only constriction of the renal arteries, either with silk ligatures or plastic clamps, induced significant elevation of blood pressure. These technics invoked hypertensive changes in 10 of 13 animals which survived 3 or more weeks after operation. In 8 of these monkeys the elevations were moderate or severe. Although constriction of the renal arteries was an effective method for producing hypertension, degree of stenosis was critical; only a slight overconstriction led to acute renal failure and death.

The remaining 4 procedures, capable of producing hypertension in such laboratory animals as the rat, rabbit, or dog, were ineffective in producing elevations in blood pressure in the monkey. The observations of Koletsky(13) in rats suggest that temporary renal artery occlusion followed by contralateral nephrectomy might have been more effective had the normal kidney been removed

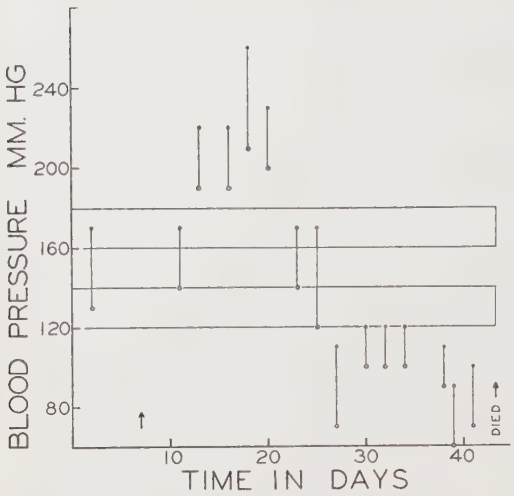

FIG. 2. Blood pressure readings following unilateral renal artery stenosis with silk ligature and contralateral nephrectomy. Cloth cuff readings; systolic (●) and diastolic (○).

at a later time when acute damage to the occluded kidney had been partially repaired. The 3 procedures employing silk perinephritis were ineffective, apparently because the silk caused very little inflammatory reaction except in those few instances in which it became infected accidentally. Examination of the wrapped kidneys at necropsy indicated that the silk had begun to disintegrate after it had been in the body about 6 months and after a year or more was discernible only as a small nodule where the purse-string tie had been made.

Blood pressure changes observed in Monkey 4900 (Fig. 2) are of interest in connection with the role of the liver in maintenance of hypertension. Raaschou and Trautner(14) found that ligation of the common bile duct in dogs with experimental renal hypertension caused a marked reduction in blood pressure often to normotensive levels. A similar series of events appears to have occurred by accident in Monkey 4900. Following application of a silk ligature to the single remaining renal artery, the monkey developed extreme hypertension. Apparently, at this point, there was bleeding into the gall bladder and bile ducts with clot formation producing complete biliary stasis. There was an abrupt drop in blood pressure to subnormotensive levels, and this suggests that in the monkey as in the dog, and possibly also in man(15,16), certain functions of the liver are essential for maintenance of elevated blood pressure at least during early hypertension.

Summary. Six methods useful in producing experimental hypertension in other laboratory animals were studied in rhesus monkeys. Two methods involving direct constriction of the renal arteries either by silk ligatures or small transparent plastic clamps resulted in a significant incidence of hypertension. Of 13 animals which survived 23 days or longer after renal artery constriction, 10 developed hypertension. The degree of constriction was critical, and a significant proportion of the animals succumbed in acute post-operative renal failure. Four other methods used successfully in rats, rabbits, or dogs were ineffective in the monkey. These included: (1) unilateral temporary renal occlusion and contralateral nephrectomy; (2) unilateral silk perinephritis; (3) unilateral silk perinephritis and contralateral nephrectomy; and (4) bilateral silk perinephritis. Administration of large oral doses of sodium chloride *per se* did not induce hypertension, nor did it increase the effectiveness of any of the above 4 procedures. Attention has been called to some of the factors which may have contributed to the failure of certain of the methods to produce hypertension in the monkey.

1. Koletsky, Simon, *Am. J. Path.,* 1950, v26, 695.
2. Page, I. H., *Science,* 1939, v89, 273.
3. Drury, D. R., *J. Exp. Med.,* 1938, v68, 693.
4. Goldblatt, H., *ibid.,* 1937, v65, 671.
5. Meneely, G. R., Tucker, R. G., Darby, W. J., and Auerback, S. H., *Ann. Int. Med.,* 1953, v39, 991.
6. Schmidt, L. H., and Genther, C. S., *J. Pharmacol. Exp. Therap.,* 1953, v107, 61.
7. Smith, Carl C., and Ansevin, A., PROC. SOC. EXP. BIOL. AND MED., 1957, v96, 428.
8. Orbison, J. L., and Peters, Evelyn, PROC. SOC. EXP. BIOL. AND MED., 1953, v83, 173.
9. Wakerlin, G. E., *Factors Regulating Blood Pressure,* Trans. 1st Conf., Josiah Macy, Jr., Fn., N. Y., 1947, p93.
10. Schroeder, H. A., and Goldman, M. L., *Circulation,* 1952, v5, 730.
11. Keys, J. E. L., and Goldblatt, H., *Arch. Ophth.,* 1938, v20, 812.
12. Frank, M. H., and Wakerlin, G. E., *Fed. Proc.,* 1954, v13, 47.

13. Koletsky, Simon, *Arch. Path.*, 1954, v58, 592.
14. Raaschou, F., and Trautner, K., *Scand. J. Clin. Lab. Invest.*, 1953, v5, 223.
15. Bradley, S. E., *Am. J. Med.*, 1948, v4, 398.
16. Spatt, S. D., and Rosenblatt, P., *Ann. Int. Med.*, 1949, v31, 475.

Received June 27, 1957. P.S.E.B.M., 1957, v96.

Porphyrin Synthesis by Mouse Harderian Gland Extracts: Sex, Age, and Strain Variation.* (23501)

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It has been observed that the Harderian glands of very young mice, 2 to 4 weeks of age, were not red fluorescent(1). It had also been shown that the red fluorescence of Harderian glands of mice of different strains varied in intensity. In some respects the Harderian glands resemble sebaceous glands in human subjects. Both of these glandular structures elaborate free porphyrins in the secretory product at some stage of development. In the adult human subject, the sebaceous glands of the skin of the face and forehead are seen as tiny red dots when illuminated with near ultra-violet light because of the red fluorescence of the sebum(2,3). However, no red fluorescent sebum was observed during the examination of the facial skin of more than 200 immature children. Thus, red fluorescent sebaceous secretions appear in the human subject at about the time of puberty, persist throughout the period of maturity, and usually decrease in older people. While the porphyrin content and red fluorescence of the Harderian glands of mice follow a similar pattern, variations in red fluorescence may occur as a result of dietary and other factors. It has been shown that the Harderian glands and the livers of mice contained an abundance of porphyrin-producing enzyme(4). Muscle-bone and brain tissues in mice contained some porphyrin-producing enzyme, but were less than 20% as effective in producing porphyrin(4).

In these experiments, an attempt was made to ascertain whether there was a variation in the porphyrin-producing enzyme content of the Harderian glands of mice of different ages and in different strains of mice of both sexes. It was found that the porphyrin-producing enzyme activity did vary with age and strain. The observations on the enzyme activity were in agreement with the previous observations on the fluorescence of Harderian glands(2,3).

Materials and methods. The Harderian glands used in this series of experiments were taken from 74 male and 78 female mice of the C_3H, C_{57}, A, and LCSa strains, varying in age from 2 to 52 weeks. The animals were killed with ether and the Harderian glands dissected out in a cold room (6°C). After removal of any excess blood, 100 mg of the tissue was weighed and placed in a mortar with a small amount of coarse grinding sand and 2 cc of 50:50 buffer-saline solution. After the tissue was homogenized, the residue was rinsed from the mortar with 3 cc of the buffer-saline solution. This was centrifuged at 2500 r.p.m. for 15 minutes and the supernatant solution was used as the enzyme extract. To each of 148 tubes (37 for each age series of tissues to be tested), 1.4 cc of 0.01 M δ-aminolevulinic acid hydrochloride buffer-saline solution was added to act as the substrate. Various amounts (0-0.8 cc) of enzyme extract, from each Harderian gland to be tested, were then placed in their respective Klett tubes. An amount of buffer-saline was added to each tube to bring the volume to 3 cc. The Klett tubes were then closed with cellophane covered corks. The tubes were incubated at 37°C for 24 hours. Concentrated HCl (6.5 cc) was then added to

* This work was supported by grants from the Anna Fuller Fund, Am. Cancer Soc., Md. Division, and N.I.H., P.H.S.

† This and the previous paper(4) were presented in partial fulfillment of the requirements for the degree of Doctor of Philosophy.

TABLE I. Age, Strain, and Sex Variation in Porphyrin Producing Enzymes of Harderian Glands of Mice.

Strain	No. mice	Age, wk	Avg μg of porphyrin produced/10 cc of substrate solution by amounts of enzyme extract indicated ♀ .2	♀ .4	♀ .8	♂ .2	♂ .4	♂ .8
C_3H	8	2	.1	5.5	6.8	.7	5.7	6.1
	8	3	1.7	7.2	8.9	2.0	6.7	10.0
	6	5	5.2	14.0	18.0	5.7	15.0	18.7
	5	8	11.7	30.5	51.5	13.7	35.0	55.7
	5	16	15.0	33.3	55.4	15.1	33.9	55.1
	5	52	14.4	33.0	52.2	16.5	35.5	54.0
Total	37							
C_{57}	8	2	—	4.0	7.0	.4	3.1	5.0
	8	3	.7	5.7	8.5	.9	5.5	7.2
	6	5	4.6	12.0	16.8	5.1	14.2	18.1
	5	8	10.7	27.6	49.8	12.8	28.9	52.4
	5	16	13.3	30.1	53.1	13.9	33.7	55.0
	5	52	12.8	32.4	51.5	12.2	30.5	52.8
Total	37							
A	8	2	—	3.0	4.6	.2	3.5	4.2
	8	3	.7	5.0	7.2	.9	5.9	8.1
	6	5	4.0	13.5	15.9	5.4	12.8	14.4
	6	8	10.2	28.0	48.5	11.5	31.1	49.0
	6	16	11.3	29.4	48.1	14.4	31.8	49.0
	5	52	11.1	30.0	50.0	12.8	33.1	48.3
Total	39							
LCSa	8	2	—	2.6	4.4	.2	3.1	4.6
	8	3	.3	4.2	5.2	.4	5.1	6.7
	7	5	3.9	10.7	13.3	3.3	9.8	15.0
	6	8	9.4	25.9	44.6	10.1	27.4	45.9
	5	16	8.3	27.4	46.8	9.0	28.7	45.0
	5	52	9.0	28.1	45.9	10.9	31.1	47.9
Total	39							

each Klett tube to give a final concentration of 25% HCl, which stopped the enzyme reaction and cleared the solution of any protein or precipitate. The tubes were then allowed to stand overnight in a cold room(6°C). A solution of hematoporphyrin hydrochloride in 25% HCl was prepared as a standard to be used in a Klett-Summerson filter photometer to determine the amount of porphyrin formed by each tissue.

Results. The variation in amount of porphyrin produced by different Harderian gland enzyme preparations is shown in Table I. The amount of porphyrin produced by the extracts of Harderian glands of mice in the 4 strains tested varied consistently with the strain and also with the age of mice. The Harderian gland extracts of mice of the C_3H strain produced the greatest amount of porphyrin, while the extracts of Harderian glands of mice of the other strains (C_{57}, A, and LCSa in this order) produced less porphyrin.

There was a great variation in the porphyrin-producing enzyme activity in the different stages of development. The Harderian glands of mice of 2 weeks of age had the lowest enzyme activity. The maximum enzyme activity was observed in Harderian glands of mice approximately 8 weeks of age. After 8 weeks, the Harderian glands showed an uniformly high enzyme content, but showed little or no increase in porphyrin-producing enzyme activity in older animals. The small variation in the amounts of porphyrin produced by the extracts of Harderian glands of mice of different sexes was not significant.

Discussion. One of the most striking observations in these experiments was the fact that enzymes for producing porphyrin are practically absent in the Harderian glands of

very young animals. As the animals become sex mature (2 to 5 weeks) the porphyrin-producing enzymes appear and increase in concentration in the Harderian gland. From this time on, there is a relatively rapid increase in porphyrin-producing enzyme activity, which reaches a practical maximum at about the age of 8 weeks. This is uniform for all of the mice of the various strains studied and is the same in both males and females. The porphyrin-producing enzyme activity then remains relatively constant until the animals are at least a year old. It is possible that if animals older than one year had been tested, that a decline in the porphyrin-producing enzyme would have been observed after the active reproductive period. While no definite correlation can be made between the sex hormones and sex activity and the fluctuations in these, the possibility exists that the porphyrin metabolism in the Harderian gland (and sebaceous glands in human subjects) may be in some way linked with sexual development and sex hormone patterns.

Summary and conclusions. 1. The porphyrin-producing enzyme activity of the Harderian glands of mice of different strains, ages, and sex was determined. 2. The Harderian gland of the C_3H strain displayed the greatest porphyrin-producing enzyme activity, while the C_{57}, A, and LCSa strains showed lower activity in the order listed. 3. The age of the animals was the most important variable. From the age of 2 weeks until the age of 8 weeks, there appeared to be a steady increase in the activity of porphyrin-producing enzyme. After this age, porphyrin-producing enzyme activity remained uniformly high in all animals up to one year of age. 4. The small differences in amounts of porphyrin produced by Harderian glands of the mice of 2 sexes was not great enough to conclude that there is a sex difference with respect to porphyrin-producing capacity in the Harderian glands of mice of the strains tested.

1. Figge, F. H. J., Strong, L. C., Strong, L. C., Jr., and Shanbrom, A., *Cancer Research,* 1942, v2, 335.

2. Figge, F. H. J., *Bull. Sch. Med. Univ. of Md.,* 1942, v26, 165.

3. Figge, F. H. J., *A.A.A.S. Research Conf. on Cancer,* 1944, p117-128.

4. Davidheiser, R. H., and Figge, F. H. J., Proc. Soc. Exp. Biol. and Med., 1955, v90, 461.

Received June 28, 1957. P.S.E.B.M., 1957, v96.

Synthesis and Demonstration of Antiserotonin Activity of 1-Benzyl-2-methyl-5-hydroxytryptamine (BAS-phenol) (23502)

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The recent demonstration(1,2) that a suitably constructed antimetabolite of serotonin may be useful in clinical treatment of essential hypertension together with the discovery, through the use of other antimetabolites of this hormone, that serotonin may play a role in the maintenance of normal mental processes (3,4,5) have focused attention on the potentialities of the antiserotonins. We have therefore continued to examine new members of this series in the hope of finding more active and otherwise more useful compounds. This report will describe the synthesis of a new analog of serotonin, *viz.* 1-benzyl-2-methyl-5-hydroxytryptamine, or 1-benzyl-2-methyl-serotonin. A demonstration will also be made that it is a very powerful antimetabolite of the hormone, and one which shows certain desirable pharmacological properties. The preceding antiserotonin which was said to be effective in hypertension was 1-benzyl-2-methyl-5-methoxytryptamine, called the benzyl analog of serotonin, or BAS. The new compound is the phenol of this methyl ether, or BAS-phenol.

The following considerations prompted synthesis and study of BAS-phenol. One prob-

* With the technical assistance of E. Van Winkle.

lem in the clinical use of BAS is that large doses bring about a feeling of drowsiness. It was thought possible that this was caused by penetration of the drug into the central nervous system. Even though it has been shown (in laboratory animals)(6) that BAS penetrates with difficulty into the brain, it was postulated that this property of the drug (difficulty in entering the brain) might be improved further by replacement of the methoxy-group with an hydroxyl. There is much empirical evidence to indicate that while methyl ethers act on the brain, the corresponding phenols do so less readily when given peripherally. In an effort then to avoid the drowsiness induced by BAS, BAS-phenol was studied.

The BAS-phenol was synthesized from BAS by cleavage of the methyl ether with pyridine hydrochloride. This method of cleavage of phenolic ethers was the only one of the several tried which was satisfactory. Thus the more common methods, which use aluminum chloride, HBr, or KOH, proved unsuccessful.

Methods. Synthesis of 1-benzyl-2-methyl-5-hydroxytryptamine (BAS-phenol) from 1-benzyl-2-methyl-5-methoxytryptamine (BAS). 1-Benzyl-2-methyl-5-methoxytryptamine hydrochloride† (7) (6.0 g) was dissolved in a solution of pyridine (30 ml) in *excess* ethanolic hydrogen chloride (150 ml of 3M). The ethanol was removed under reduced pressure and the resultant viscous syrup was warmed at 75° and 12 mm pressure for 20 minutes to complete the solvent removal. The flask containing the residue was then provided with an air condenser and heated at 200 ± 5° for 30 minutes. The yield decreased with longer heating. When cooled, the melt was dissolved in water (400 ml) and filtered. 6N Sodium hydroxide was added until the pH reached 9 (indicator paper) when the product, which initially separated as an oil, crystallized. Seeding was useful in bringing this about. Excess alkali caused the product to redissolve. The material was filtered, washed with water, and dried *in vacuo*. The yield of base was 4.4 g, 86%, m.p. 199-201°. Recrystallization from aqueous pyridine gave a constant m.p. of 201-203°

Anal. calc'd for $C_{18}H_{20}N_2O$:	C, 77.11; H, 7.19; N, 9.99
Found:	C, 77.01; H, 7.19; N, 9.86

The hydrochloride of BAS-phenol was obtained by suspension of the above base in boiling absolute alcohol (5 ml per g) and addition of ethanolic hydrogen chloride to effect solution. When cooled, the hydrochloride crystallized in an over-all yield of 76%, m.p. 244-245°.

Anal. calc'd for $C_{18}H_{21}N_2OCl$:	C, 68.24; H, 6.68; N, 8.84
Found:	C, 68.50; H, 6.90; N, 8.70

Testing for antiserotonin activity. The ability to prevent the pressor action of serotonin in dogs was used for testing for antiserotonin activity. Details of these methods have been given previously(8,9). The animals were calibrated as to their pressor responses to serotonin and graded doses of BAS-phenol, dissolved in Ringer's solution, were injected intravenously each followed by a serotonin challenge. The smallest dose was thus determined which would protect against the pressor response to serotonin‡ administered 15 minutes after the analog. The various precautions described earlier with respect to times of injection and methods of measurement were strictly heeded.

Antiserotonin potency by the oral route was determined as follows. Dogs were calibrated for their pressor responses to intravenous serotonin as described previously(8,9). They were then fed BAS-phenol for 4 days. The drug was given once daily, mixed with the food. On the fourth day of feeding, just after the final dose of drug, the animals were anesthetized again with Nembutal, and again challenged with serotonin intravenously.

Results. Antiserotonin activity of BAS-phenol as measured by intravenous injection into dogs. By the intravenous route, BAS-phenol was very effective in antagonizing the pressor action of serotonin. In fact, it was

† We wish to thank Merck and Co. for gifts of this compound.

‡ Serotonin was generously provided by Dr. K. E. Hamlin, Abbott Laboratories.

TABLE I. Rises in Arterial Blood Pressure of Dog 32 Given Serotonin* Intravenously after Injection of BAS-phenol.

Amt of analog (mg/kg)	Interval between analog and serotonin (min.)	Rise in pressure (mm of Hg)
0	—	52
.035	15	52
.120	15	28
.120	90	48
.350	15	6

* Challenging dose of serotonin was 0.12 mg of 5-hydroxytryptamine creatinine sulfate/kg body wt. BAS-phenol was 1-benzyl-2-methyl-5-hydroxytryptamine hydrochloride.

the most active compound encountered thus far among all the serotonin analogs examined in this laboratory. Data from a typical experiment are shown in Table I, where it can be seen that protection against 0.12 mg of serotonin per kg was afforded by 0.35 mg of BAS-phenol per kg. Similar results were found in 4 other dogs.

Comparison of BAS-phenol with BAS in this intravenous test showed that the phenol was considerably more active. Thus, 3.0 mg of BAS per kg of body weight was required by the intravenous route (avg of 3 dogs) whereas 0.3 mg of BAS-phenol was needed for similar protection (avg of 5 dogs). Furthermore, the phenol exerted its maximal effect within 15 minutes (results from 4 dogs). For these reasons (high potency and rapid action) BAS-phenol recommended itself over previous compounds where an intravenously administered antiserotonin was desired.

Protection of dogs by the oral route against the pressor action of serotonin. The data of Table II show that doses of serotonin which had elicited good rises in arterial pressure in the unprotected animals, failed to do so after the ingestion of BAS-phenol. In the 6 experiments summarized there, this phenol at about 1 mg per kg per day protected all of the dogs, but at one-tenth of this dose there was no protection. By the oral route, therefore, BAS-phenol was approximately equal in potency to BAS. Previous trials(9) done under identical conditions (some of them on the same dogs as used for the BAS-phenol) had shown that BAS gave protection at 1 mg per kg per day by the oral route. Clearly then BAS-phenol was approximately 10 times more active than BAS by the intravenous route, but approximately equal to it in potency by the oral route. These comparisons of course apply only to the pressor test with serotonin and may not necessarily be valid for other test systems.

Serotonin-like action of BAS-phenol. When anesthetized dogs were injected intravenously with solutions of BAS-phenol, a sharp rise in arterial pressure was always observed (6 of 6 dogs). This was in contrast to the failure to find such rises with BAS when tested under identical conditions(9). The rise in arterial pressure was rapid and short-lived and in these respects resembled the response to serotonin. The pressures always returned to the original values within one to 2 minutes. Quantitative comparisons (in these 6 dogs) of graded doses of serotonin with graded doses of BAS-phenol showed that serotonin-creatinine sulfate was about 10 times more active than BAS-phenol (as the hydrochloride). However, one dog was found in which BAS-phenol was 3 times as active as serotonin.

The rise in pressure elicited by BAS-phenol was probably serotonin-like because it could be prevented by prior treatment of the animal with BAS. BAS is known to protect against serotonin but to be unable to do so against adrenaline or even against tryptamine(10). The BAS-protected dog is thus a very specific test object for serotonin-activity. A dog which, unprotected, had given a rise in arterial pressure of 26 mm of Hg following intravenous injection of BAS-phenol (0.6 mg per kg) was fed BAS (3 mg per kg per day) for 3 days.

TABLE II. Rises in Arterial Pressure of Various Dogs Given Serotonin Intravenously before and after Ingestion of BAS-phenol.

Oral dose of analog (mg/kg/day)	Serotonin response*	
	Before analog (mm of Hg)	After analog (mm of Hg)
3.2	30	6
1.4	36	7
1.1	52	8
1.2	23	8
.14	36	41
.11	52	40

* Challenging dose of serotonin was 0.06-0.12 mg of 5-hydroxytryptamine creatinine sulfate/kg body wt.

When challenged again with BAS-phenol (same dose) the rise in pressure was insignificant (8 mm of Hg). Tryptamine (1.1 mg/kg), however, elicited a full response (80 mm of Hg). This experiment was repeated in another dog with similar results.

Specificity. In the intravenous test with dogs as described above BAS-phenol was a remarkably specific antagonist of serotonin. It did not antagonize the action of adrenaline, noradrenaline, or even of tryptamine. Data illustrating these points are being published elsewhere(10).

Toxicity of BAS-phenol. Acute and chronic toxicities for adult mice (10 for each dose level) given BAS-phenol intraperitoneally were determined by the methods previously described for other members of this series(9, 11). All animals were killed by a single injection of 80 mg/kg and all survived a single injection of 40 mg/kg. A daily dose of 40 mg/kg for one week killed 2 of 10. Twenty mg/kg per day for one month was well tolerated. No animals died and none lost weight or showed changes which could be detected by visual inspection of the organs on autopsy.

Summary. A method was described for synthesis of 1-benzyl-2-methyl-5-hydroxytryptamine involving cleavage of the methyl ether of 1-benzyl - 2-methyl - 5-methoxytryptamine (BAS) by heating with pyridine hydrochloride. The phenol so produced exhibited powerful antiserotonin action either orally or intravenously in dogs. In the intravenous test it was the most potent antiserotonin thus far encountered. It also had considerable serotonin-like activity.

1. Woolley, D. W., and Shaw, E., *Science,* 1956, v124, 34.

2. Wilkins, R. W., *New England J. Med.,* 1956, v255, 115.

3. Woolley, D. W., and Shaw, E., *Proc. Nat. Acad. Sci.,* 1954, v40, 228.

4. ———, *Brit. Med. J.,* 1954, vol. ii, 122.

5. Gaddum, J. H., In *Ciba Foundation Symposium on Hypertension,* 1954, Little, Brown & Co., Boston, Mass.

6. Woolley, D. W., Van Winkle, E., and Shaw, E., *Proc. Nat. Acad. Sci.,* 1957, v43, 128.

7. Shaw, E., *J. Am. Chem. Soc.,* 1955, v77, 4319.

8. Woolley, D. W., and Shaw, E., *J. Pharm. Exp. Therap.,* 1953, v108, 87.

9. Shaw, E., and Woolley, D. W., *ibid.,* 1956, v116, 164.

10. Woolley, D. W,, and Shaw, E., *ibid.,* 1957, in press.

11. Shaw, E., and Woolley, D. W., PROC. SOC. EXP. BIOL. AND MED., 1956, v93, 217.

Received July 15, 1957. P.S.E.B.M., 1957, v96.

Effect of a Brain Extract on Turnover Rate of Serum Cholesterol.*† (23503)

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At high levels of serum cholesterol a definite reduction will usually be achieved in human subjects with the feeding of an alcoholic extract of defatted brain(1). This closely simulates the effect of oral beta-sitosterol(2). However, when low normal levels of serum cholesterol already exist, a significant reduction of the total serum cholesterol may not be achieved with similar dosage. Thus the question arises whether any effect on cholesterol metabolism occurs in such cases, and further whether any effect exists in the absence of dietary cholesterol which had been used in our earlier chicken experiments(3). The following experiments were performed in an effort to elucidate the effect of this material upon the endogenous cholesterol metabolism in the experimental animal.

Methods. Chick experiment. An 8-week-old cockerel, on a diet of 100 g per day of starter mash, received an intravenous injec-

* This work was supported by grants from Amer. Heart Assn., Eli Lilly Foundation and the U.S.P.H.S.

† This work was presented in part at 28th Annual Meeting of Amer. Heart Assn. Oct. 1955.

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tion of 51 mg of 1-C^{14} sodium acetate containing 1 mc/meq. Blood samples were drawn at least twice weekly in the same postprandial state for 8 weeks. From the 21st day to the 34th day 10% of the mash was supplanted by the cerebroside extract§ previously described(1). At each bleeding at least 6 ml of blood was drawn and oxalated, the plasma was removed and duplicate 1.5 ml aliquots were precipitated and refluxed for 20 minutes in 30 ml of 3:1 alcohol:ether. The filtrate and washings were pooled, evaporated and the residue saponified under nitrogen in 10% alcoholic KOH. The unsaponifiable matter was then extracted 3 times with petroleum ether, the extract evaporated under N_2 and transferred to a small centrifuge tube with a few ml of ether. The ether was then allowed to evaporate under N_2, and the residue dissolved in 0.5 ml of absolute ethanol. The supernatant was then treated with digitonin by a modification of the method of Hellman *et al.* (4). The digitonide was then transferred as an ether slurry to preweighed planchets 3.47 cm in area and allowed to dry for several hours at room temperature and for 15 minutes at 110°C. After determining the constant weight of the digitonide, the samples were counted in a windowless gas flow counter, the counts per minute being corrected to infinite thickness of the digitonide from an experimentally determined curve.

Dog experiment. A 16 kg mongrel dog was injected intravenously with 42 mg of I-C^{14} sodium acetate containing 1 mc/meq and, 14 days later, 0.8 mc of $Na_2HP^{32}O_4$ was injected intravenously. The constant daily diet of 207 g Purina Lab. chow plus 228 g of a commercial dog food was fed throughout, except that 40 g per day were replaced by the brain extract from the 20th to the 29th day of the experiment. Oxalated blood samples were obtained at 2- to 5-day intervals and the plasma obtained by centrifugation. Duplicate 5 ml samples were precipitated in 100 ml of a 1:1 alcohol:acetone solution which was brought to the boiling point on a water bath and filtered. The filtrate was taken to dryness and the residue extracted 4 times with small volumes of boiling petroleum ether. This extract was again taken to dryness, saponified, and the digitonide precipitate obtained and again washed as described by Hellman, Rosenfeld and Gallagher(4), except that it proved necessary to wash the digitonide twice with ethanol at 0°C to avoid contamination of the digitonide by P^{32}. In addition, duplicate samples of 1.5 ml of plasma were precipitated in 10 ml of trichloracetic acid for determination of the radioactive phosphorus incorporated in the phospholipid. The supernatant was discarded and the precipitate washed 3 times with 5% trichloracetic acid before being extracted 4 times with 3 ml of boiling ethanol after the method of Schneider(5). The phospholipid was then isolated by the technic of Kennedy (6). After the final washing, 4 ml of the resulting 2:1 ethanol:CCl_4 extract was placed in a centrifuge tube and taken to dryness. The residue was then digested in an aluminum block with 0.4 ml of 10N H_2SO_4 until charred, and treated with H_2O_2 until clear. This was then brought to a 5.5 or 10 ml volume, and an aliquot of 0.5 ml plated on a planchet for immediate counting while wet. A 5 ml aliquot was then used for determination of phosphorus by the method of Gomori(7). The planchets were counted in the gas flow counter and the counts per minute per microgram of phosphorus determined. Samples of feces were collected on the 11th and 23rd day. These were homogenized in a Waring blendor with an equal volume of hot acetone, centrifuged and the supernatant collected. The residue was further extracted with hot acetone 3 times, and then taken to dryness at room temperature, ground in a mortar and pestle and placed in a desiccator until constant weight was obtained. This weight is referred to as the "dry weight" of the feces, though it is recognized that in addition to being dry it is relatively free of easily soluble lipid. An aliquot of the acetone extract was then saponified in 10% alcoholic KOH for 20 minutes and the unsaponifiable matter extracted with 3 washings of 150 ml petroleum ether. An appropriate aliquot of the petroleum ether extract was then dried down in a preweighed centrifuge cone, redissolved in absolute alcohol and pre-

§ We are grateful to Dr. David Klein of Wilson Laboratories who supplied the brain extract employed.

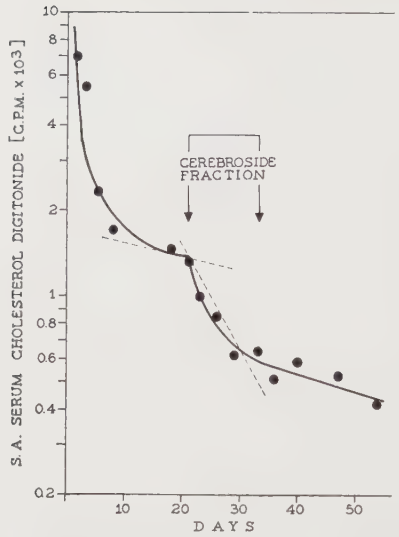

FIG. 1. Biological decay of C^{14}-cholesterol digitonide derived from serum of a chick which received a single intravenous injection of C^{14}-acetate at zero time. Dotted lines indicate the approximate slope of this decay curve immediately before and during period of administration of cerebroside fraction (from day 21 to 34).

cipitated quantitatively by digitonin by the method of Schoenheimer and Dam(8). The resulting precipitate was washed thrice with cold ethanol, once with ether:dioxane(1:1) and twice with ether, and the amount of sterol estimated from the digitonide which was dried for 30 minutes at 110°C and brought to constant weight in a desiccator. The total unsaponifiable matter was obtained gravimetrically by weighing the residue from an appropriate aliquot of the ether extract taken to dryness in a tared flask. The total unsaponifiable matter and digitonin precipitable sterol in the feces were then expressed as percent of dry weight of the fecal residue.

Results. In Fig. I the specific activity of the cholesterol digitonide precipitated from the serum lipids is plotted logarithmically against time. It can be seen that the rapid phase of the biological decay of labeled cholesterol is largely finished by the fifth day. After this the decay curve approaches a straight line asymptote, the slope of which is proportional to the turnover rate of the plasma cholesterol, provided the total plasma cholesterol level is constant(9). From previous experience we know that the cholesterol level of the plasma is but slightly influenced by the administration of brain extract when the bird is receiving a plain mash diet without oil or cholesterol supplement(10).

During the period of administration of the cerebroside fraction, there is a sharp increase in slope, the mean negative slope during this period being virtually twice that of the immediate pre-treatment period and 3 to 4 times that of the post-treatment period. The post-treatment slope cannot be said to differ from that anticipated had the more rapid decay period not intervened.

Fig. 2 illustrates that the same change in rate of turnover of serum cholesterol is induced in the dog during the 9 day period of administration of the extract. Serum cholesterol levels were obtained before and during administration of this cerebroside fraction by the Abell-Kendall technic(11) and the values during the control period ranged from 89-100 mg%, during the treatment period from 84-108 mg%. It was concluded that there was no significant change in the total serum cholesterol. As in the chicken, the turnover rate of the serum cholesterol did increase during the treatment period.

In Fig. 3 the decay curve for the P^{32} labeled phospolipid is presented. The points approximate a straight line, though a slight increase in turnover rate of lipid phosphorus may be perceptible after the 4th day. There was no alteration in slope of the magnitude seen with the C^{14} cholesterol. Again, there was no significant trend in the serum lipid phosphorus concentration, which varied between 6 and 7.1 mg%.

Fecal sterols were measured per unit dry weight of feces on 2 occasions, which may be considered representative of control and treatment periods. The results presented in Fig. 2 indicate that the total unsaponifiable matter per gram of weight (24 mg) was increased under treatment (35 mg) as was the weight of digitonin precipitable sterols per gram of dry weight of feces (6.4 mg to 14.0 mg). Thus, per unit of dry weight, the fecal sterols were increased by more than 2-fold. The specific activity of the digitonide on these 2 days was 27 and 44 counts per minute (corrected to infinite thickness). Thus, during

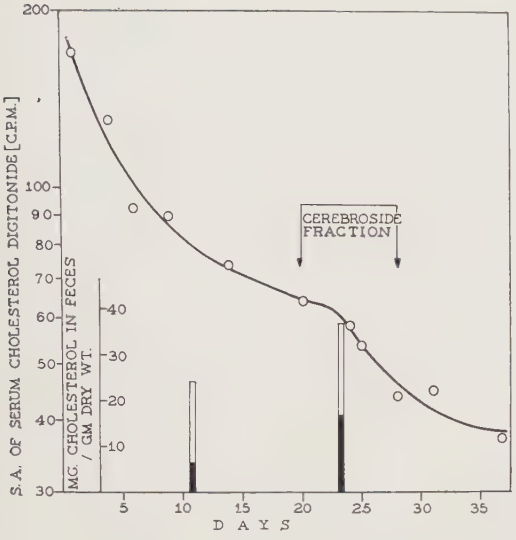

FIG. 2. Biological decay of C^{14}-cholesterol digitonide derived from serum of a dog which received a single intravenous injection of C^{14}-acetate at zero time. Total height of the bar represents milligrams of unsaponifiable matter per gram of dry weight of the feces; shaded portion represents milligrams of digitonin-precipitable sterol per gram of dry weight of feces on the days indicated. Cerebroside fraction was administered from day 20 to 29.

the feeding of the brain extract the specific activity of fecal sterols was 60% higher even though the specific activity of the serum cholesterol was, in fact, 30% lower than during the earlier period.

Discussion. These studies in the chick and the dog indicate that administration of this brain extract, rich in cerebrosides, will increase rate of turnover of the serum cholesterol, even in situations where no great change in total serum cholesterol obtains. There is ample evidence that an elevated total serum cholesterol will be reduced by this material(1,3), though no great change in serum cholesterol was expected in these experiments. Had a slight decrease occurred here, changes in the total specific activity of the serum cholesterol (S.A. $\times$ mg%) would have been even more dramatic. The turnover rate of phospholipid is but slightly influenced by the administration of the extract, there being no change comparable to that of the serum cholesterol. However, since the serum phospholipids include lecithin sphingomyelin and cephalins, an increased turnover of a minor component, such as sphingomyelin or a cephalin, cannot be excluded. With this reservation, we may conclude that the primary effect on the serum lipids is exerted upon the cholesterol rather than the phospholipids.

The nature of the brain extract effect seems to be an increase in turnover rate of the serum cholesterol. In the face of a constant serum level of cholesterol this implies an increased rate of removal from the serum of cholesterol higher in specific activity than that replacing it. Furthermore, this extract promotes an increased excretion of fecal sterols. Even more striking is the increase in their specific activity in the face of a falling specific activity of the serum cholesterol, whence it must be derived. These data strongly suggest a deflection of cholesterol from its entero-hepatic cycle. Similar observations on quantitative changes in fecal sterols have been made by Carroll *et al.*(12) who fed rats cerebrosidic fatty acids.

The mode of action of this extract is not yet entirely clear. A large percentage of the material remains in the intestinal tract to be excreted in the feces, thus increasing the bulk of the feces. The sterol seems to be intimately bound with this cerebroside residue, for it can be completely separated only with great difficulty. On the other hand, x-ray diffraction studies performed by the Physicochemical Research Division of the Lilly Research Laboratories, through Dr. R. E. Shipley, to whom we are deeply indebted for this kindness, have indicated that no mixed crystals can be distinguished when cholesterol and phrenosin are crystallized together in the same system productive of mixed cholesterol-beta-sitosterol crystals(13). It would appear that this cerebrosidic material somehow binds the

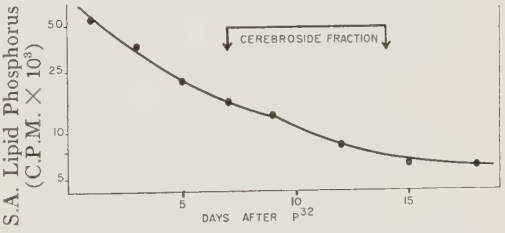

FIG. 3. Biological decay curve of P^{32} phospholipids after a single intravenous injection of $Na_2HP^{32}O_4$ on 14th day of the experiment in Fig. 2, designated as day zero on the abscissa here.

intraintestinal cholesterol or otherwise inhibits its reabsorption into the portal system. This leads to an increase in the turnover rate of serum cholesterol and, if it is elevated, a reduction in its level.

Summary. In radioisotope studies in the chick and the dog, the turnover rate of the serum cholesterol, as measured by the biological decay of endogenously labeled C^{14} cholesterol, was substantially increased by the feeding of a cerebroside-rich extract of mammalian brain. Much less influence was found on the turnover rate of P^{32} labeled phospholipids. Some evidence is presented to suggest that the increased turnover rate of serum cholesterol is produced by an increased fecal excretion of endogenous cholesterol.

The authors are indebted to Mrs. Irene Carr for technical assistance and to Dr. George LeRoy for reviewing the manuscript.

1. Jones, R. J., *J. Lab. Clin. Med.,* 1956, v47, 261.
2. Best, M. M., Duncan, C. H., Van Loon, E. J., and Wathen, J. D., *Circulation,* 1954, v10, 201.
3. Jones, R. J., Kraft, S. C., Huffman, S., Balter, E. L., and Gordon, R. B., *Circulation Res.,* 1953, v1, 530.
4. Hellman, L., Rosenfeld, R. S., and Gallagher, T. F., *J. Clin. Invest.,* 1954, v33, 142.
5. Schneider, W. C., *J. Biol. Chem.,* 1945, v161, 293.
6. Kennedy, E. P., *ibid.,* 1953, v201, 399.
7. Gomori, G., *J. Lab. and Clin. Med.,* 1942, v27, 955.
8. Schoenheimer, R., and Dam, H., *Z. physiol. chem.,* 1933, v215, 59.
9. Zilversmit, D. B., Entenman, C., and Fishler, M. C., *J. Gen. Physiol.,* 1943, v26, 325.
10. Gordon, R. B., Kraft, S. C., and Jones, R. J., *J. Lab. and Clin. Med.,* 1953, v41, 583.
11. Abell, L. L., Levy, B. B., Brodie, B. B., and Kendall, F. E., *J. Biol. Chem.,* 1952, v195, 357.
12. Carroll, K. K., and Noble, R. L., *Canad. J. Biochem. and Physiol.,* 1956, v34, 981.
13. Davis, W. W., *Tr. New York Acad. Sci.,* Ser. II, 1955, v18, 123.

Received July 15, 1957. P.S.E.B.M., 1957, v96.

Effect of Gluco- and Mineralocorticoid Adrenal Steroids on Fluid and Electrolytes of Fasted Adrenalectomized Dogs.* (23504)

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Certain hormones of the adrenal cortex apparently are concerned with regulating the distribution of water and electrolytes between body compartments(1-11). Workers in this laboratory employing the potent steroid 2-methyl-9α-fluorohydrocortisone, which has both gluco- and mineralocorticoid activity demonstrated that adrenalectomized dogs verging on prostration from insufficiency could be restored to health and vigor within 48-60 hours despite withholding of food and water. Thus, dogs exhibiting severe symptoms evidently possess stores of water and salt in their cells and tissues adequate to effect apparent restoration of extracellular fluid and electrolyte to normal without necessitating additional supplies from exogenous sources(10-11). If cortical hormones are lacking such animals are unable to mobilize and freely shift these vital substances and death results. However, when the appropriate type of corticoid is administered i.v. to these critically ill dogs, large volumes of water, Na, Cl and K are withdrawn from unidentified stores and shifted to the extracellular and intravascular compartment. As a result, serum Na and Cl are elevated to normal, the inspissated blood becomes diluted and the marked accumulations of blood urea nitrogen and serum K decline to control values. These fluid and electrolyte transfers are accompanied by a

* Acknowledgement is made to The National Science Foundation, The Upjohn Co., Merck, Sharp & Dohme and Ciba Pharmaceutical Products, Inc., for financial support. 1-dehydrohydrocortisone was supplied by the Schering Corp.

TABLE I. Effect of 1-dehydrohydrocortisone and Desoxycorticosterone on Serum Electrolytes of Fasted Dogs Recovering from Adrenal Insufficiency.

Dog No.	Day bled	Condition*	Dose, mg/day	Body wt, kg	BP, mm Hg	Blood urea N, mg %	Hb, g %	Hmct, %	RBC 10^6, mm^3	Blood sugar, mg %	Serum electrolytes (mEq/l) Na	Cl	K
						1-dehydrohydrocortisone (free alcohol)							
3–27†	1	N	Initial	12.02	108	16.0	10.90	32.2	4.94	80.0	146.0	116.9	4.22
	5	S	15	11.79	60	112.0	15.25	32.6	5.15	88.0	130.0	105.2	8.94
	6	A	15	10.88	80	66.6	13.47	33.9	4.56	82.5	142.5	116.2	4.97
	7	R	0	10.40	84	22.0	12.28	30.0	4.23	110.0	148.5	120.2	4.49
1–27	1	N	Initial	12.70	108	16.7	12.91	36.8	6.45	88.5	142.0	114.0	3.91
	7	S	15	12.25	78	78.0	12.91	42.8	7.22	93.0	132.0	111.4	8.29
	8	A	15		125	28.9	12.28	38.9	6.26	105.0	—	—	—
	9	A	15	10.88	120	22.4	11.20	36.4	6.27	106.0	138.0	114.1	3.96
	10	R	0	10.43	120	45.4	10.20	34.1	5.23	124.5	142.5	120.3	3.79
2–52	1	N	Initial	21.25	102	18.5	13.33	35.6	5.06	86.0	145.5	113.5	4.48
	10	M	15	21.00	60	41.0	18.04	42.8	6.22	80.5	137.0	107.5	6.18
	11	A	15		77	33.2	16.01	43.1	5.92	93.0	—	—	—
	12	A	15	19.88	84	63.8	13.89	36.8	4.84	92.0	130.5	104.9	5.20
	13	R	0	18.14	86	61.2	11.82	36.6	4.88	100.0	139.0	103.7	5.26
2–49	1	N	Initial	15.99	116	23.0	11.48	33.8	4.68	85.0	143.5	117.2	4.38
	4	M	15	14.97	67	52.0	17.36	48.0	6.61	72.0	137.0	103.2	6.62
	5	A	15		78	62.4	13.89	41.6	5.54	93.5	130.0	107.3	4.82
	6	R	15	13.84	107	74.6	12.51	36.3	4.92	98.5	137.5	105.3	4.62
	7	R	0	13.26	112	90.5	12.05	33.7	4.87	100.0	138.0	107.3	3.96
2–50	1	N	Initial	12.02	111	13.8	17.59	50.5	6.96	84.0	147.0	116.8	3.58
	11	S	15	11.45	54	100.0	21.88	57.3	9.67	73.5	131.5	93.6	6.76
	12	A	15	11.23	69	55.0	21.04	59.1	8.53	76.5	—	—	—
	13	R	15	11.00	93	33.4	20.14	57.1	9.48	92.0	144.0	117.6	5.20
	14	R	0	10.54	97	57.4	18.64	52.9	8.06	95.5	143.5	110.5	4.71
2–54	1	N	Initial	15.31	112	12.3	13.75	36.1	5.21	83.0	143.5	112.8	4.54
	7	S	25	14.85	52	55.6	19.84	49.4	7.26	82.0	133.0	100.0	7.64
	8	A	25	13.95	66	37.0	16.01	48.8	6.86	93.5	—	—	—
	9	R	25	13.61	94	38.8	15.43	42.6	6.35	105.5	141.5	102.5	4.46
	10	R	0	13.26	106	38.0	15.43	38.3	5.78	119.8	141.5	101.2	4.48
4–30†	1	N	Initial	16.25	100	16.4	11.94	35.5	5.16	74.0	144.5	119.0	3.90
	6	S	2	15.88	60	47.0	15.25	37.8	6.10	81.0	129.0	113.5	7.40
	7	M	2	15.20	78	19.6	14.20	38.2	5.63	92.0	—	—	—
	8	M	0	15.08	68	14.9	13.50	38.0	5.07	92.0	126.0	102.5	6.39

(Continued on next page)

TABLE I (continued).

Dog No.	Day bled	Condition*	Dose, mg/day	Body wt, kg	BP, mm Hg	Blood urea N, mg %	Hb, g %	Hmct, %	RBC 10^6, mm^3	Blood sugar, mg %	Serum electrolytes (mEq/l) Na	Cl	K
						Desoxycorticosterone (free alcohol)							
5–47	1	N	Initial	12.13	118	18.3	14.50	41.6	6.54	77.5	147.0	116.2	4.30
	10	S	300	11.57	45	81.0	21.00	56.6	9.72	80.0	129.5	104.5	6.36
	11	M	200	11.23	68	74.0	19.84	51.5	8.98	96.5	—	—	—
	12	M	0	10.68	50	95.0	20.50	49.7	7.55	89.0	136.5	113.1	4.95
6–44	1	N	Initial	14.51	106	21.5	14.00	37.0	5.45	83.5	145.0	115.0	4.14
	11	M	300	14.17	73	47.0	18.94	48.7	8.99	81.0	124.5	91.5	6.33
	12	A	200	13.49	64	36.2	17.59	40.5	6.68	94.0	—	—	—
	13	A	0	13.04	64	50.0	16.46	37.0	6.79	108.0	145.0	110.0	5.36

* Condition of animal: N = normal, S = severe symptoms, M = mild symptoms, A = active and vigorous, R = complete recovery.
† Dogs 3-27 and 4-30 were fasted 48 hr, the remaining animals 72 hr.

profuse diuresis with extensive renal elimination of Na, Cl, K and water. Symptoms disappear and activity and vigor return.

The interpretation offered for these and other similar data was reiteration of a suggestion originally made over 20 years ago(1-3), *viz.*, that when adrenal cortical hormones are lacking, water and certain electrolytes are immobilized in cells and tissues hence are no longer capable of being freely shifted from intra- to extracellular and intravascular spaces no matter how great the need for such redistribution. Thus adrenal corticoids are not only necessary for preventing undue wastage of salt and water in the urine of animals developing insufficiency owing to a functional renal defect (first pointed out by Loeb *et al.* (12), and confirmed by Harrop *et al.*(13), Harrison and Darrow(14) and others), but perhaps of even greater importance for the well-being of the organism, are necessary for maintenance of the normal dynamic equilibrium and free exchange of water and electrolytes between the fluid compartments of the body.

The present experiments were designed to compare the efficacy of relatively specific gluco- *vs.* mineralocorticoids for restoring health and vigor to adrenalectomized dogs presenting serious insufficiency symptoms while held without access to food and water during the restoration period. Two steroids were chosen for this purpose: 1-dehydrohydrocortisone (1-dehydro F or prednisolone, Schering), and desoxycorticosterone (DOC, Ciba). The former compound possesses strong glucocorticoid activity but is weak in Na-retaining properties; in fact it is regarded by some as natriuretic(15-18). Long term adrenalectomized dogs require at least 20 mg/day to maintain a normal serum electrolyte pattern(19). DOC, although devoid of glucocorticoid activity except when given in relatively enormous doses, is a potent mineralocorticoid which readily induces serum Na and Cl retention but stimulates renal excretion of K.

Methods. Nine dogs were used; they had been adrenalectomized for periods ranging from 1-5 years and all had been previously

TABLE II. Effect of 1-dehydrohydrocortisone and Desoxycorticosterone on Serum and Urine Electrolytes of Fasted Adrenalectomized Dogs Recovering from Adrenal Insufficiency.

Dog No.	Fasting period, hr	Total dose, mg	Serum electrolyte changes during fast (mEq/l)			Total urine electrolyte excretion during fast (mEq)			Urine vol recovery period, cc
			Na	Cl	K	Na	Cl	K	
			1-dehydrohydrocortisone						
1–27	72	45	+10.5	+ 8.9	−4.50	45.09	56.33	52.10	880
3–27	48	30	+18.5	+15.0	−4.45	58.65	16.26	43.01	985
2–52	72	45	+ 8.5	− 3.8	− .92	107.60	96.00	99.80	1385
2–49	72	45	+ 8.0	+ 4.1	−2.66	77.83	75.40	103.10	1250
2–54	72	75	+ 8.5	+ 1.2	−3.16	27.71	34.19	98.90	1035
4–30	48	4	− 3.0	−11.0	−1.01	83.75	92.14	37.22	518
2–50	72	30	+12.0	+16.9	−2.05	22.10	18.63	39.63	984
			Desoxycorticosterone						
5–47	48	500	+ 7.0	+ 8.5	−1.41	1.61	3.77	23.89	305
6–44	48	500	+20.5	+18.5	− .97	3.29	6.21	38.02	574

+ = Increase in serum electrolytes. − = Decrease in serum electrolytes.

studied through several bouts of adrenal insufficiency and restoration to normal health by adrenal steroid therapy. When not in use for experiments they were maintained by daily i.m. injections of 0.5 mg of DCA plus daily NaCl supplements (2 g) in the food. DCA and salt were withdrawn when an experiment started and normal or control values for the various blood constituents established. These are recorded in Table I under the headings Day Bled and Initial Blood Sample. The dogs were then permitted to develop severe insufficiency. The interval required for signs of adrenal failure to appear varied from 4 to 11 days as indicated in the table under Day Bled. Immediately following withdrawal of the blood sample the blood pressure was determined, the bladder emptied and an i.v. injection of either l-dehydro F or DOC administered. The quantity of steroid used per dog/day is listed in Table I under dosage and was not the same for all dogs. The animals were placed in metabolism cages and deprived of food for 48-72 hours. Injections were repeated at 12-hour intervals during the fasting period and total dose varied from 2-25 mg/day. The free alcohol of l-dehydro F and DOC was used and the daily amount given in divided doses morning and evening. The material was prepared fresh each day and solubilized in a vehicle consisting of 25% absolute ethanol, 25% propylene glycol and 50% water. The preparation of this vehicle is important if the steroids are to remain in solution. Not more than 125 mg of the DOC were given i.v. at a time; the full 250 mg morning and afternoon doses were each administered as 2 injections about 30 minutes apart. The total amount of fluid injected during the fasting period was balanced by withdrawal of a similar volume of blood for study. Upon completion of the fast and remission of symptoms the arterial pressure, body weight and blood constituents were again determined and the experiment terminated. The quantity of urine, Na, Cl and K eliminated each 24 hours was measured; the data are recorded in Table II.

Results. A. Effect of l-dehydrohydrocortisone (l-dehydro F) on the Internal Distribution of Water and Electrolytes. Data obtained from 7 animals are given in Tables I and II. At time of injection the dogs exhibited severe symptoms of adrenal insufficiency *e.g.*, loss of weight, low arterial pressure, hemoconcentration, elevated serum K, low serum Na and Cl. They were weak, spastic and had refused food for 16-24 hours. Two (1-27 and 3-27) had experienced severe cardiac episodes probably due to hyperpotassemia. Within a few hours after hormone administration the blood pressure began to rise and generally within 24 hours the animals displayed normal activity and vigor. Hemodilution as revealed by decreases in hemoglobin, hematocrit and erythrocyte counts was evident. The effect of the steroid on the blood urea nitrogen was variable and much less impressive. It was reduced from the high level characteristic of

adrenal insufficiency to control values only in 2 dogs (3-27 and 4-30). In dog 1-27 it declined from 78 to 22.4 mg% within 36 hours but increased again during the last 13 hours of the fast. The remaining animals failed to show a return of this metabolite to control levels. In a previous study using 2-methyl-9α-fluorohydrocortisone the writers demonstrated rapid lowering of blood urea nitrogen in fasted adrenal insufficient dogs. Dog No. 4-30 which received a much smaller dose (4 mg over 48 hours), was unable to elevate blood pressure or serum Na and Cl (Tables I and II). However, this small amount of l-dehydro F was sufficient in this case to lower blood urea nitrogen and induce renal excretion of large quantities of Na, Cl and K plus 518 cc of urine. Blood sugar increased moderately during the fast; the highest level reached was 124 mg% which represents an increase of 36 mg% over the initial value. Since this steroid is a potent glucocorticoid the rise in blood sugar is not surprising. The serum Na increased to normal in all dogs except the one receiving the lowest dosage. This increase ranged from 8 to 18.5 mEq/l. Serum Cl did not invariably follow the Na in response to therapy since the serum concentrations rose sharply in 3 dogs (Nos. 1-27, 3-27 and 2-50), but fell in 2 others (Nos. 4-30 and 2-52). The 2 remaining dogs presented rises in serum Cl of 4.1 and 1.2 mEq/l. Serum K declined in all of the treated animals; the maximum decrease was 4.5 and the minimum was 0.92 m Eq/l (Table II).

Large quantities of water, Na, Cl and K were excreted by the kidneys of these fasted dogs. Table II records the total amounts eliminated together with the increases of serum Na and Cl during recovery despite continued loss of these electrolytes in the urine and the hemodilution taking place. These data indicate a profound regulatory action of the glucocorticoid upon electrolyte and water shifts between the fluid compartments of the body. The quantities of these vital substances thus redistributed are such that it seems reasonable to assume they were chiefly derived from intracellular sources and can be shifted to extracellular and intravascular spaces only when sufficient amounts of the appropriate type of adrenal cortical hormone are present. There was some indication that dogs which exhibited the smallest increases in serum Na and Cl during revival from insufficiency tended to excrete the largest quantities of these electrolytes in the urine (Table II, dogs 2-52, 4-30 and 2-49).

B. Effect of Desoxycorticosterone (DOC). Results of 2 tests in which this steroid was used for reviving fasted dogs from adrenal insufficiency were not impressive despite the massive (500 mg) dosage employed, when compared with those obtained with much smaller doses of l-dehydro F. It is known, of course, that DOC is effective in restoring adrenal insufficient animals which take in food and water. High dosage was deemed necessary to insure the animal's ability to survive the prolonged fast and lack of salt. DOC in the dosage used raised the serum Na and Cl; one dog showed an increase of 20.5 and the other 7 mEq/l of Na within 48 hours. This increased serum Na concentration appeared to be due in part to renal excretion of water and retention of Na. Serum Cl was also elevated but serum K declined (Table I). However reestablishment of the normal serum electrolyte picture had no significant effect on either arterial pressure which remained low or blood urea nitrogen which remained high. Hemoconcentration persisted but some dilution did occur. Adrenal insufficiency symptoms were much improved in one dog (6-44), but were present in the other animal at the end of the fasting period. Moderate increases in blood sugar were apparent in both dogs and may possibly have been due to conversion of excess DOC to a glucocorticoid type steroid.

Discussion. DOC was much more effective than the glucocorticoid in stimulating renal retention of Na and Cl; however, both induced loss of K and a diuresis which in the case of DOC consisted of practically Na and Cl-free water. The important differences in activity between these steroid types as employed in these experiments are as follows:

DOC—500 mg/day	1-dehydro F—15 mg/day
1. Marked renal retention Na and Cl.	1. Profuse renal excretion Na and Cl.

2. Increase in serum Na presumed due in part to exer'n "salt-free" water.	2. Incr. in serum electrolytes presumed due to internal redistribution.
3. Persistence of low blood pressure.	3. Rise in blood pressure to control or near control values.
4. Persistent hemoconcentration.	4. Hemodilution present.
5. Activity and vigor less than normal in one dog, definite insufficiency symptoms present in the other.	5. Return of activity and vigor in all dogs within 24 hr.

Thus the recovery response of the fasted adrenal insufficient dogs was distinctly in favor of the glucocorticoid. The animals receiving l-dehydro F, no matter how serious their condition when injected, exhibited dramatic recovery.

Data in Table III represent the net urinary excretion of Na, Cl and K by adrenalectomized dogs developing insufficiency (data taken from the classical metabolic study of Loeb *et al.*(12)), compared with the total renal elimination of these electrolytes by 3 representative cases of dogs recovering from insufficiency while receiving l-dehydro F. Loeb's animals were offered the usual daily ration and allowed water *ad libitum* during the 6-7 days following adrenal removal and during the interval insufficiency symptoms were developing. The dogs in our experiments, although deprived of exogenous sources of fluid and electrolyte, excreted quantities of Na, Cl and K during recovery which compare favorably with total quantities lost in the urine by the dogs of Loeb *et al.* over a 6-7 day period during which time they were eating normally for the first 4-5 days. It seems reasonable to assume that fluid and electrolyte changes induced in these fasted glucocorticoid-treated dogs during revival from insufficiency are too great to be accounted for other than by an outflux of Na, Cl, K and water from cells, including probably, bone and collagenous tissue.

As an alternative explanation it might be assumed since water was withheld during recovery on steroid treatment that the copious amount of water excreted during this period was in sufficient excess of Na to adequately account for elevation of the latter in the plasma. Thus the increase in plasma Na could be explained by a renal mechanism without evoking internal redistribution of the cation. However, in an extensive series of experiments (unpublished) the writers have observed that adrenalectomized dogs, not in insufficiency but deprived of food and water for 48 hours, when injected i.v. with 2-methyl FF, exhibit a rise of plasma electrolytes despite a sharp increase in plasma volume, decreased hemoglobin, hematocrit, RBC and a severe diuresis with continued loss of water and electrolyte.

Another point of interest is the apparent correlation between ability of l-dehydro F (also 2-methyl FF(10-11)) to restore blood pressure and to induce internal redistribution of fluid and electrolyte. Our data offer no suggestion as to which of these events was primary and which secondary; the fact remains, nevertheless, that DOC was unable to elevate arterial pressure in the fasting animal despite relative enormous dosage and marked Na retention, whereas the glucocorticoids do so in

TABLE III. Total Renal Excretion of Electrolytes of Dogs Developing Adrenal Insufficiency over 6-7 Days Compared with That of Animals Fasted 48-72 Hours during Recovery on Glucocorticoid Therapy.

	Adrenalectomized dogs. No treatment* Fed 4-5 days†			Adrenalectomized dogs. Fasted 48-72 hr Glucocorticoid therapy		
mEq/l	Dog 1 (7)	Dog 2 (6)	Dog 3 (6)	Dog 2–52	Dog 1–27	Dog 2–49
Na	–64.80	–111.23	–102.54	–107.60	–45.09	– 77.83
Cl	–68.60	– 63.21	– 56.81	– 96.00	–56.33	– 75.40
K	–40.28	– 9.80	– 93.38	– 99.80	–52.10	–103.10

* Data taken from metabolic study of adrenal insufficiency by Loeb *et al.*(12). No. in parentheses indicate No. of days between gland removal and sacrifice of the animals.

† Dogs 1 and 3 ate usual ration for 4 days; dog 2 ate for 5 days according to Loeb *et al.*

– = mEq output over intake of Na, Cl and K.

the face of continued sodium loss. Previous studies(20-21), demonstrated that adrenalectomized dogs can be maintained symptom-free for weeks with serum Na and Cl levels either as low or lower than occur in severe insufficiency. The low serum electrolyte animals remain active and vigorous and exhibit normal blood pressure so long as glucocorticoids are administered in adequate amounts. These experiments imply that a possible direct effect of the steroids on the vascular system may be of paramount importance.

Summary. 1) l-dehydrohydrocortisone induced rapid revival of fasted adrenalectomized dogs from severe insufficiency. Disappearance of symptoms and return of activity and vigor was accompanied by restoration of normal values of arterial pressure and serum electrolytes. Hemodilution was evident and a profuse diuresis occurred with marked renal loss of Na, Cl, K and fluid. Desoxycorticosterone, in doses of 500 mg/day was much less effective in restoring activity but sharply increased serum Na and Cl, an increase which in part seemed due to excretion of relatively "salt free" water. There was renal retention of Na and Cl, persistent hemoconcentration and failure of the blood pressure to rise above the low level characteristic of adrenal insufficiency. 2) The fluid and electrolyte changes of the glucocorticoid-treated dogs are presumed to be due to an outflux of Na, Cl, K and water from cells including probably bone and collagenous tissue. Although an apparent correlation exists between the ability of l-dehydrohydrocortisone to shift fluid and electrolytes and to restore arterial pressure, some of the data suggest that a possible direct effect of this type of steroid on the peripheral vasculature may be of primary importance in revival from insufficiency.

1. Swingle, W. W., Pfiffner, J. J., Vars, H. M., and Parkins, W. M., *Am. J. Physiol.,* 1934, v108, 144, 428.

2. ———, *ibid.,* 1934, v107, 259.

3. ———, *ibid.,* 1937, v119, 684.

4. Gaudino, M., and Levitt, M. F., *J. Clin. Invest.,* 1949, v28, 1487.

5. Flanagan, J. B., Davis, A. K., and Overman, R. R., *Am. J. Physiol.,* 1950, v160, 89.

6. Woodbury, D. M., *ibid.,* 1953, v174, 1.

7. Hills, A. G., Chalmers, T. M., Webster, G. D., and Rosenthal, O., *J. Clin. Invest.,* 1953, v32, 1236.

8. Luft, R., Sjogren, B., Ikkos, D., Ljungren, H., and Tarukoski, H., *Recent Progress in Hormone Research,* 1954, v10, 425. Academic Press, N. Y.

9. Woodbury, D. M., and Koch, A., PROC. SOC. EXP. BIOL. AND MED., 1957, v94, 720.

10. Swingle, W. W., Brannick, L. J., and Parlow, A. F., *ibid.,* 1957, v94, 156.

11. Swingle, W. W., Brannick, L. J., and Parlow, A. F., *Schweiz. med. Wschr.,* 1957, v87, 152, Supple. to No. 14.

12. Loeb, R. R., Atchley, D. W., Benedict, E. M., and Leland, J., *J. Exp. Med.,* 1933, v57, 775.

13. Harrop, G. A., Soffer, L. J., Ellsworth, R., and Trescher, J. H., *ibid.,* 1933, v58, 17.

14. Harrison, H. E., and Darrow, D. C., *Am. J. Physiol.,* 1939, v125, 631.

15. Bunim, J. J., Pechet, M. M., and Bollet, A. J., *J.A.M.A.,* 1955, v157, 311.

16. Pechet, M. M., and Bartter, F. C., *J. Clin. Endocrinol. and Metab.,* 1955, v15, 85.

17. Stafford, R. O., Barnes, L. E., Bowman, B. J., and Meinzinger, M. M., PROC. SOC. EXP. BIOL. AND MED., 1955, v89, 371.

18. Chart, J. J., Hetzel, N., and Gaunt, R., *ibid.,* 1956, v91, 73.

19. Swingle, W. W., Brannick, L. J., and Parlow, A. F., *Endocrinol.,* 1957, v60, 658.

20. Swingle, W. W., Collins, E., Barlow, G., and Fedor, E. J., *Am. J. Physiol.,* 1952, v169, 270.

21. Swingle, W. W., Barlow, G., Collins, E., Fedor, E. J., Welch, W. J., and Rampona, J. M., *Endocrinol.,* 1952, v51, 353.

Received July 24, 1957. P.S.E.B.M., 1957, v96.

Modification of Infectious Bovine Rhinotracheitis (IBR) Virus in Tissue Culture and Development of a Vaccine. (23505)

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A virus isolated in bovine kidney tissue culture, and identified as the cause of IBR, has been described(1,2). Since the initial appearance of this disease in Colorado in 1950(3), it has been observed in almost every western state, and as far east as Ohio. All reports indicate that this condition is of major economic importance to the livestock industry (3-5). The propagation of the etiological agent in tissue culture made development of a vaccine feasible. This paper shows that rapid passages of IBR virus in bovine kidney tissue culture and subsequent selection by terminal dilutions have resulted in a virus so modified that it could be injected without production of disease, but with formation of antibodies.

Material and methods. Virus. One strain of IBR virus, Colorado I, was employed for the modification studies. This strain was isolated in bovine embryo kidney tissue culture roller tubes from respiratory tissues of 2 cows in the acute phase of IBR. *Tissue Culture Procedures.* Bovine embryo kidney tissue culture in roller tubes was prepared from trypsinized kidney cortex 8-9-months-old fetuses. The nutrient medium consisted of 0.5% lactalbumin hydrolysate, 5-10% horse serum, and Earle's basic salt solution containing 200 units of penicillin and 200 μg of streptomycin/ml. A more detailed description of preparation of these cultures has been given(2). To obtain larger quantities of virus, 5 g of bovine embryonic kidney cortex were minced to 1-2 mm size, suspended in 500 ml of the above-mentioned medium in Povitsky bottles, and shaken for 3-4 days at 35°C. These bottles were usually inoculated with 1 ml of infected tissue culture fluid containing not less than 4 logs of virus, shaken for an additional 4 days, and the fluids harvested. Regular passages of the virus were made by harvesting the infected tissue culture fluid and transferring 0.2 ml amounts to new cultures only after a complete cytopathogenic effect (CPE) occurred on the cells. Rapid passages consisted of transfers of infected tissue culture fluid to new cultures in 0.2 ml amounts per tube at 24-48 hour intervals, when approximately 25-50% of the cells exhibited a cytological change. To select the virus particles which were present in the majority at a given passage level, terminal dilutions were performed. Ten-fold dilutions of the infected tissue culture fluids were prepared in tissue culture medium without horse serum, and at least 3 kidney tissue culture tubes were inoculated with each dilution. The fluid from the culture inoculated with the highest dilution which still produced a CPE was harvested, 10-fold dilutions again prepared, and these dilutions inoculated into new tissue cultures. *Neutralization Test.* Equal amounts of serum or serum dilutions were mixed with equal amounts of virus in a final concentration of 100 to 1,000 $TCID_{50}$ (50% tissue culture infectious dose). This mixture was incubated for 2 hours at 37°C, 0.2 ml inoculated into each of 3-6 kidney tissue culture tubes, and observed for several days. The serum neutralization effect was recorded when approximately 100 $TCID_{50}$ of virus was observed in the virus control titration carried out simultaneously. *Cattle.* Cattle 4 months of age or older, regardless of breed or sex, were used for the laboratory part of this work. They were generally held in isolation for at least 2 weeks prior to inoculation, and daily temperature records as well as other observations were made to insure that the animals were normal before use. The susceptibility of the cattle to IBR was determined by absence of neutralizing antibodies in their serum. In tests for immunity, cattle were challenged intranasally with 1-2 ml of virulent (low passage tissue culture fluid) virus. The virus was sprayed into the nostrils with a syringe and needle partially pinched off at the end to give a spraying effect. Daily temperatures were recorded and

observations made for other signs of illness such as anorexia, depression, increased respiration, nasal discharge, hyperemia of the nasal mucosa, and the presence of serofibrinous exudate covering the surfaces of the nasal mucosa. While a moderate fever of slight duration alone might be suggestive of a reaction to challenge, no response was considered definite until one or more of the other signs of illness in addition to fever were also observed.

Results. 1. *Modification of Virus.* In experiments to modify the virulence of the IBR virus, a series of rapid passages was initiated. To obtain a large number of virus particles, the 7th regular passage of the agent was selected as starting material. After 21 consecutive rapid passages, 3 terminal dilutions were made with a portion of this 21st passage material. A larger amount of virus was then prepared by inoculating several tissue culture tubes with the 3rd terminal dilution virus and harvesting the tissue culture fluid after a complete CPE occurred. This virus is henceforth referred to as RP 21. Using a portion of the unpurified 21st rapid passage material, 19 additional rapid passages were made. This material was also passed through 3 terminal dilutions. Again, a pool of virus was prepared by inoculating tissue culture tubes with the 3rd terminal dilution virus (RP 40).

Inasmuch as a practical vaccine for cattle, employing a modified virus, would have to be administered parenterally, the intramuscular route was selected as a preliminary means of determining any reduction in virulence which might have occurred at any particular point during tissue culture passages. It had been noted that when a susceptible calf was inoculated intramuscularly with $10^{5.2}$ $TCID_{50}$ in 1 ml of the first regular passage of the original infectious material in tissue culture (T.C. #1), the animal responded after an incubation period of 2-3 days with a fever up to 104.8°F, increased respiration, anorexia, and depression. A similar response, with fever of 105.8°F, anorexia, and increased respiration, was observed when a calf was inoculated with 5 ml of the 15th regular passage in bovine kidney tissue culture titering $10^{6.4}$/ml. Accordingly, a susceptible calf was inoculated with 1 ml of tissue culture fluid containing 10^{8} $TCID_{50}$ of RP 21. Another calf was inoculated with 1 ml of tissue culture fluid containing $10^{6.2}$ $TCID_{50}$ of RP 40. Temperature response and other observations were made daily for 3 weeks, following which each animal was challenged intranasally with virulent virus. Serum samples were obtained at the time of inoculation, and again prior to challenge. Following inoculation with RP 21, a febrile response occurred within 24 hours, reaching 106°F on the 3rd day. No other signs of illness, such as anorexia or increased respiration, could be observed. The calf inoculated with RP 40 developed no fever or other signs of illness. No antibodies could be demonstrated in the pre-inoculation serum samples of these animals, although in each instance neutralizing antibody titer from 1:9 to 1:38 occurred when 2-3 week serum samples were tested. None of the animals showed any signs of illness when challenged intranasally with virulent IBR virus.

2. *Tests in Cattle.* To determine whether results similar to those above with RP 40 could be reproduced, several larger batches of virus were prepared either in trypsinized or minced bovine kidney tissue cultures using RP 40 as seed virus. (a) *Young Experimental Cattle.* One or more calves weighing from 350-500 lb were inoculated intramuscularly with each lot of virus prepared, using a total of 18 animals. Serum samples were taken at the time of inoculation, and again 3-4 weeks later. When the 2nd sample was taken, the animals were challenged intranasally with a virulent virus capable of producing typical IBR in 1 or more susceptible control animals inoculated simultaneously. None of the animals showed signs of IBR, all developed neutralizing antibodies, and resisted a challenge with virulent virus (Table I). (b) *Titration of Tissue Culture Virus in Cattle.* All cattle used in the above experiments were inoculated with 4 or more logs of virus, but provided no indication of the minimum $TCID_{50}$ of virus necessary to immunize an animal. To use this modified virus as a vaccine, it was necessary to determine the minimum amount of virus required to immunize an animal. Tenfold dilutions of RP 40 virus were prepared in tissue culture media, and for each dilution

TABLE I. Response of Cattle Inoculated Intramuscularly with Modified IBR Virus (RP 40).

No. of cattle	$TCID_{50}$ inoc. (logs)	Febrile response	Other signs of illness	Antibody response Pre	Antibody response Post	Challenge response
2	4 –5	0/ 2*	0/ 2	0/ 2	2/ 2†	0/ 2
10	5.1–6	1/10‡	0/10	0/10	10/10	0/10
6	6.1–7	2/ 6‡	0/ 6	0/ 6	6/ 6	0/ 3§

* Numerator = No. of animals responding; denominator = No. of animals used.
† Antibody titer range from 1:3 to 1:19. ‡ A fever above 104°F lasting for only 1 day.
§ Only 3 of the 6 inoculated cattle were challenged.

1 calf was inoculated intramuscularly. The exact amount of virus in the 1 ml inoculum for each animal was determined by a simultaneous titration in tissue culture. It was found that an animal inoculated with $10^{4.2}$ $TCID_{50}$ of virus developed a definite antibody response and resisted challenge. However, $10^{3.2}$ $TCID_{50}$ of virus produced a very low antibody response which did not prevent entirely a reaction to challenge with virulent virus. Because of questionable immunity developed in this animal, it was estimated that $10^{3.7}$ $TCID_{50}$ of virus represented a minimum immunizing amount. Therefore, in all subsequent experiments, animals were inoculated with at least 10 times the minimum immunizing amount. (c) *Beef Cattle.* Since laboratory tests with the modified virus were conducted with cattle younger than those in which the disease ordinarily occurs, and since they were held in isolation rooms not subject to marked climatic changes, the modified virus was tested in heavy beef-type animals under simulated field conditions. To facilitate the use of the modified virus in the field, 2 lots of RP 40 virus were prepared and lyophilized in glass vials. This lyophilized vaccine proved to be quite stable, and when held at refrigerator temperature (4°C) for 8 months, no loss in titer could be demonstrated. With the co-

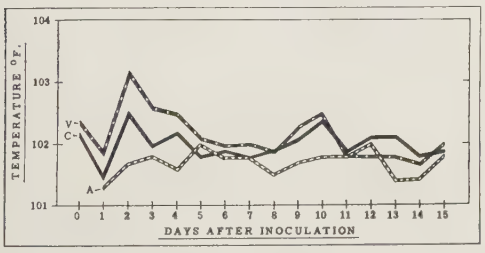

FIG. 1. Composite graph of temperature response of 30 cattle vaccinated with RP 40 virus (V), compared with temperature curves of 21 uninoculated contact controls (C) and 14 uninoculated area controls (A).

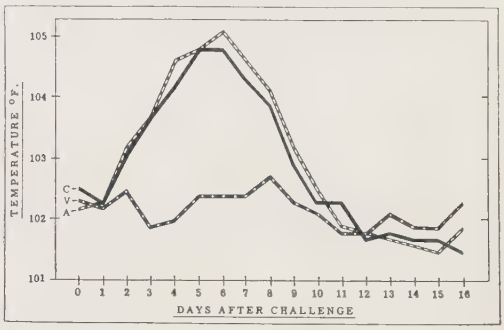

FIG. 2. Composite graph of temperature response of 30 vaccinated cattle (V), 21 contact controls (C), and 14 area controls (A) following challenge with virulent IBR virus

operation of Dr. John W. Kendrick *et al.* of the University of California School of Vet Med at Davis(6), and Dr. Rue Jensen *et al.*, School of Vet Med, Colorado State University, a total of 30 animals was used for vaccination. Each animal was inoculated intramuscularly with 2 ml of vaccine containing at least 50,000 $TCID_{50}$ of virus restored from the lyophilized state with distilled water. As controls, a total of 21 animals was held in contact with the vaccinated cattle, and 14 additional control animals were held in the same general area, but not in direct contact with them. All animals were observed daily and temperatures recorded. After 3 weeks, all the animals were challenged intranasally with virulent virus. Serum samples taken from all cattle before vaccination had no demonstrable neutralizing antibodies against IBR. The average daily temperatures for each group of cattle (Fig. 1) show that febrile response in the vaccinated group differed little from that of the controls. No signs of illness could be observed. In contrast, however, following challenge with virulent virus, the 2 control groups had a marked rise in fever although the vaccinates remained normal (Fig. 2). No signs of illness were ob-

TABLE II. Comparison between RP 40 Virus and Low Passage Virulent Virus Inoculated Intranasally into Cattle.

Inoculum	Calf No.	$TCID_{50}$ of virus inoc. × 1000	Results		
			Clinical	Antibody titer	Challenge
Modified virus (RP 40)	157	200	Normal	0	F,N,H,L,D*
	158	200	"	0	N,H,L
	159	200	"	1:3	Normal
	160	200	"	1:7	"
Virulent virus (T.C. #2)	152	320	F,H,L,N	1:23	
	153	320	F,R,D,A,H,L,N,W	1:38	
	150	3.2	F,R,D,A,H,L,N,W	Autopsied	
	155	.32	F,R,D,A,H,L,N,W	1:23	
	151	.032	Normal	0	F,N,H,L,R

* F, fever; R, increased respiration; D, depression; A, anorexia; H, nasal hyperemia; L, serofibrinous exudate; N, nasal discharge; W, loss of wt.

served in the vaccinates, except a mild fever in 1 animal, while 33 of the 35 controls developed other signs of IBR in addition to fever, and 2 died. (d) *Dairy Cattle.* Since one of the characteristic signs of illness following infection with IBR virus is abrupt cessation of milk production in the dairy animal(4,7), it was desirable to learn whether this modified virus would produce such an effect. For 5 days prior to inoculation, morning and evening temperatures and milk production were recorded for each of 5 Holstein cows. Three animals, selected at random, were inoculated intramuscularly with 2 ml of vaccine containing 2 million $TCID_{50}$ of virus. The 2 remaining animals were inoculated with a tissue culture placebo to serve as contact controls. Serum samples were taken from each animal prior to inoculation, and again 3 weeks later. For 2 weeks following inoculation, milk production and temperatures were again recorded. No change in milk production, or any other signs of illness, was noted in these animals. The virus-inoculated cattle developed antibodies, while controls remained negative.

3. *Comparison between Modified Virus and Virulent Virus by Intranasal Inoculation of Cattle.* Previous work(2,5) indicated that only by intranasal inoculation could all the signs of illness observed in the field be reproduced in experimental cattle. Therefore, this route was employed to obtain additional evidence of modification. Four susceptible animals were each inoculated intranasally with 2 ml of infected tissue culture fluid containing 200,000 $TCID_{50}$ of RP 40 virus, as determined by a simultaneous titration in tissue culture. At the same time, 10-fold serial dilutions of low tissue culture passage (T.C. #2) virulent virus of the same strain were prepared. One or more susceptible animals were inoculated intranasally with 2 ml of this material for each dilution. A simultaneous titration in tissue culture was performed. Serum samples were taken from the animals at time of inoculation, and again 3 weeks later. At the end of the 3-week period of observation, those animals showing no response to the first inoculation were challenged intranasally with virulent virus. The results of this test (Table II) show that as much as 200,000 $TCID_{50}$ of modified virus produced no signs of illness, but only 2 of the 4 animals developed demonstrable antibodies and resisted challenge. However, as little as 320 $TCID_{50}$ of virulent virus produced definite disease and antibodies. When 2 additional calves were inoculated intranasally with $10^{6.5}$ and $10^{6.8}$ $TCID_{50}$ of modified virus, respectively, mild hyperemia and a small amount of serofibrinous exudate, accompanied by a febrile response to 104.6°F for 1 day, were noted.

4. *Non-transmissibility of Virus Inoculated Intramuscularly.* In the work described above, control animals in direct contact with cattle inoculated intramuscularly with modified virus did not develop antibodies, and were susceptible when challenged. In addition, a calf held in contact with an animal inoculated in the same manner with virulent virus also remained susceptible. Since the virus apparently did not spread, deliberate attempts were made to determine whether virus could be recovered from animals inoculated by this route.

Blood and nasal washings were collected at daily intervals for 4-10 days from 5 cattle inoculated with modified virus. Specimens were also collected for as long as 27 days from 1 animal inoculated with virulent (T.C. #1) virus. The blood was defibrinated and inoculated in 0.5 ml amounts into each of several bovine kidney tissue culture tubes. After 3-5 days' incubation, the fluid was removed and new media placed in the tubes. Nasal washings were obtained by flushing the nostrils several times with tissue culture media, using approximately 20 ml of fluid. The washings were centrifuged at 5,000 rpm for 20 minutes, and the supernatant inoculated in 0.2 amounts into each of several tissue culture tubes. If yeast or mold contamination occurred in the tubes, the fluid was removed, filtered, and re-inoculated into additional tissue culture tubes. The inoculated tubes of blood and nasal washings were observed for 10 days, and if no changes in the cells occurred, they were either discarded or blind passages made. No IBR virus could be recovered from the animals tested.

Discussion. In attempts to develop a modified live virus vaccine for IBR, rapid passages were initiated to select virus variants with a high multiplication rate in bovine kidney tissue culture, assuming that such virus particles might also have a different disease-producing potential, and therefore conceivably possess less virulence. When the variant particles exceed the number of original particles, it is possible to segregate them by methods such as the terminal dilution technic.

In the data presented, it was shown that after 40 rapid passages, followed by 3 terminal dilutions, a strain of virus was recovered which was avirulent. Even in tests with a rather large group of animals inoculated intramuscularly with this strain, no response occurred except for a transient rise in temperature in some instances. This lack of response might have been due partly to the unnatural route of inoculation, since even when virulent virus was inoculated intramuscularly a febrile response, increased respiration, anorexia and depression occurred, but other signs of IBR such as nasal discharge, hyperemia, and serofibrinous exudate on the nasal mucosa were absent. Following intranasal inoculation with as little as 320 $TCID_{50}$ of virulent virus, however, all of these signs of disease could be produced. Definite evidence that RP 40 virus has been greatly modified is shown by the fact that, in contrast to virulent virus, when 625 times more RP 40 virus was sprayed into the nasal passages, not only were no signs of illness produced, but half of the animals failed to develop antibodies, indicating that this modified virus had lost much of its infectivity. It is possible that when large amounts of RP 40 virus are administered intranasally, a local reaction may occur without the development of generalized illness.

The possibility of reversion to virulence by natural back-passages always arises in connection with a modified live virus vaccine. However, it has been demonstrated that this virus did not spread from the vaccinated cattle to susceptible contacts, and could not be reisolated from nasal washings and blood of vaccinated animals, which makes this possibility very unlikely.

The inability to recover virus from intramuscularly inoculated cattle, and the fact that it requires relatively large amounts of modified virus to immunize an animal raises the question as to whether it multiplies or acts as pre-formed antigen. However, regardless of the mode of action, when 5,000 or more $TCID_{50}$ of RP 40 virus is inoculated intramuscularly, an immune response against IBR regularly occurs.

Summary. 1. A modified IBR virus was produced by rapid passages and terminal dilutions in bovine embryo kidney tissue culture. Its avirulence was demonstrated by intramuscular and intranasal inoculation of susceptible cattle. 2. Modified virus inoculated intramuscularly elicits an immune response which protects cattle against challenge with virulent virus. 3. The virus did not spread from intramuscularly-inoculated cattle to susceptible contacts, and could not be reisolated from blood or nasal washings of vaccinated cattle. 4. This modified virus prepared in tissue culture can be successfully lyophilized, thus allowing its use as a vaccine for prevention of IBR disease.

1. Madin, S. H., York, C. J., and McKercher, D. G., *Science,* 1956, v124, 721.

2. York, C. J., Schwarz, A. J. F., and Estela, L. A., PROC. SOC. EXP. BIOL. AND MED., 1957, v94, 740.

3. Miller, N. J., *J.A.V.M.A.,* 1955, v126, 463.

4. McKercher, D. G., Moulton, J. E., and Jasper, D. E., *Proc. U. S. Livestock Sanitary Assn.* 58th Meeting, 1954, 260.

5. Jensen, R., Griner, L. A., Chow, T. L., and Brown, W. W., *ibid.,* 1955, 189.

6. Kendrick, J. W., York, C. J., and McKercher, D. G., *ibid.,* 1956, 155.

7. Schroeder, R. J., and Moys, M. D., *J.A.V.M.A.,* 1954, v125, 471.

Received July 29, 1957. P.S.E.B.M., 1957, v96.

Development of Phenylalanine Hydroxylase in Liver of the Rat.* (23506)

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Inability to convert phenylalanine to tyrosine, a reaction catalyzed by the enzyme system phenylalanine hydroxylase, is known to occur as an hereditary metabolic abnormality. Jervis(1) and Udenfriend and Bessman(2) have reported that individuals with phenylpyruvic oligophrenia almost completely lack the ability to oxidize phenylalanine, while Jervis(3) has shown that postmortem specimens of livers of two patients with this disease were inactive in *in vitro* assays for phenylalanine hydroxylase. Recently, detailed biochemical observations on liver biopsy(4) and autopsy (5) material have confirmed and extended his findings.

Since other enzymes whose deficiencies in postnatal life are associated with disorders of incomplete metabolism are known to be essentially inactive in fetal life(6)—glucose-6-phosphatase(7) and the enzymes of tyrosine oxidation(8)—it seemed worthwhile to determine if the enzyme system, phenylalanine hydroxylase, demonstrates a similar developmental pattern.

Methods. Fetal, newborn, and adult rats of the Long-Evans strain were used. Fetal animals were studied as close as possible to predicted term. They and newborn rats were killed by decapitation; adult rats by a blow to the head. Livers were removed in the cold room (temperature 2 to 4°C) and immediately placed on cracked ice. Fetal as well as newborn livers were pooled to obtain adequate samples. The livers were homogenized in the cold with a Teflon homogenizer in 2 volumes of ice-cold 0.15 M KCl. Homogenates were spun in a Spinco Model L centrifuge for 30 min. at 100,000 x gravity. The soluble fractions were assayed directly or after 3 hours of dialysis against 0.15 M KCl in 0.01 M phosphate buffer at pH 7.0. Freezing at −15°C for several days resulted in no loss of activity. The soluble fractions of livers (1.0 ml) were shaken at 35°C in air in a reaction mixture containing 2 μM of L-phenylalanine, 150 μM of phosphate buffer, pH 7.0, 5 μM of nicotinamide, and 2 μM of reduced diphosphopyridine nucleotide; final volumes were 1.75 ml. The reaction was stopped after 20 to 60 minutes incubation by addition of 1.75 ml of 10% trichloroacetic acid. Following centrifugation, formed tyrosine was measured colorimetrically(9) on suitable aliquots of the deproteinized solution. Control values obtained with reaction mixtures incubated without phenylalanine were subtracted in each case. Preparations of known activity were concurrently assayed to ensure satisfactory experimental conditions. Nitrogen was determined on aliquots of the liver soluble fractions by the Ma-Zuazaga modification of the micro-Kjeldahl method(10).

Results. Phenylalanine hydroxylase activity in dialyzed soluble fractions of livers of

* This work was supported in part by grants from Natl. Inst. of Arthritis and Meabolic Diseases, N.I.H., Assn. for Aid of Crippled Children; and Damon Runyon Memorial Fund.

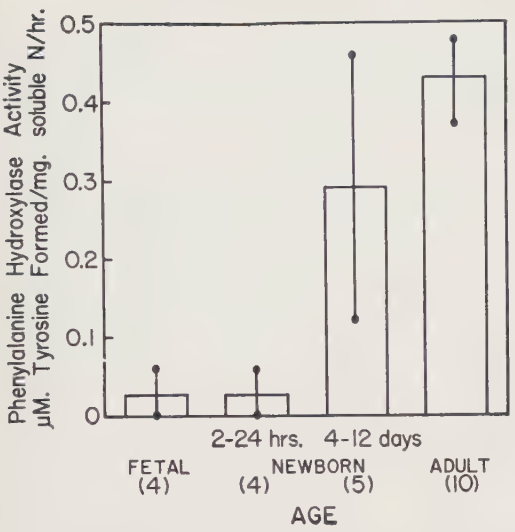

FIG. 1. Phenylalanine hydroxylase activity of rat liver in relation to age. Dialyzed soluble fractions of liver were assayed as described in the text. Height of bars represents the mean; lines drawn through points represent range of experimental values obtained. Figures in parentheses denote number of experiments performed. Values greater than 0.4 μM tyrosine/mg N correspond to complete conversion of the 2 μM of phenylalanine added to the reaction mixtures.

fetal, newborn, and adult rats are summarized graphically in Fig. 1.

Fetal rats. Experiments with dialyzed soluble fractions of fetal liver indicated that conversion of phenylalanine to tyrosine was absent or negligible at this stage of development. Samples were incubated for one hour; under these conditions 8% or less of the added phenylalanine was hydroxylated.

Newborn rats. Enzyme activity of dialyzed preparations of livers of rats less than 24 hours old was within the range of fetal preparations; not more than 8% of added phenylalanine was converted to tyrosine. These essentially inactive soluble fractions could not be stimulated by addition of larger amounts (up to 6.0 μM) of pyridine nucleotides in either oxidized or reduced form. In the dialyzed soluble fractions of livers of rats 4 to 12 days old, complete conversion of the added phenylalanine to tyrosine was observed in one of the assays. The other 4 converted 35 to 55% of the phenylalanine.

Adult rats. Phenylalanine hydroxylase activity of dialyzed soluble fractions of livers

TABLE I. Effect of Dialysis on Activity of Phenylalanine Hydroxylase in Liver Soluble Fractions of Rats.*

Age	μM tyrosine/ml sol. frac. Before dialysis	After dialysis for 3 hr	% activity lost
Adult	1.84	1.34	27
"	2.37	1.24	48
"	2.02	1.68	17
"	2.11	1.23	42
Fetus	0	0	
"	.15	.05	67
Newborn (23 hr)	.85	.16	81
" (4 days)	2.18	1.14	48

* Incubation time for liver soluble fractions of adult rats—20 min.; of fetal and newborn rats—60 min.

obtained from adult animals of both sexes, following periods of incubation of 20 to 60 minutes, showed a conversion capacity of 50 to 90% of added phenylalanine in 20 minutes. Within one hour conversion was complete in 8 of 10 observations. In the remaining 2, conversion was 80% and 95% complete.

Effect of Dialysis. Recent studies by Kaufman(11,12) on the mechanism of this reaction in liver extracts have indicated the presence of a hitherto unrecognized cofactor. Since this cofactor is presumably dialyzable, the influence of dialysis on the activity of the soluble fraction was studied (Table I). The activity of adult liver preparations was decreased 17 to 48% after 3 hours of dialysis. Soluble fractions of livers of fetal and newborn rats were also tested before and after dialysis. Loss of activity in these preparations was considerably more marked, ranging from 48 to 81%. Nevertheless, the activity of undialyzed preparations of fetal livers never exceeded a conversion of 8%, and in one experiment, no activity was found prior to or following dialysis. Thus the low activity of fetal and newborn liver preparations is not due primarily to inactivation by dialysis, but the more marked lability of these preparations does suggest that they contain less of a dialyzable component (or a component more readily inactivated during dialysis) than those of adult animals.

Discussion. The results indicate that phenylalanine hydroxylase activity is absent or negligible in the livers of fetal rats near term

and in newborn rats less than 24 hours old. Within a few days following birth the enzyme system approaches the level of activity observed in adult animals.

It has recently been established that conversion of phenylalanine to tyrosine in liver extracts requires participation of 2 discrete enzymes(13), as well as reduced pyridine nucleotide(14) and the unidentified cofactor mentioned previously. One of the components of the enzyme system can be found in tissues which do not hydroxylate phenylalanine, while the second enzyme, which is specific, has been found only in liver. This second specific enzyme is now known to be the component which is lacking in the livers of individuals with phenylketonuria(4,5). Recent experiments in this laboratory have shown† that the non-specific components of the overall reaction are present in fetal liver, and that inactivity of these preparations can also be traced to a deficiency in the specific enzymic component of liver. The study of phenylalanine hydroxylase provides a striking example of a situation wherein enzymic deficiency encountered in an hereditary metabolic disease, phenylpyruvic oligophrenia, occurs as the normal pattern in the fetal mammal. Further studies of this enzyme in the fetal animal may contribute information on the pathogenesis of the enzymic defect of phenylpyruvic oligophrenia.

Summary. Development of phenylalanine hydroxylase in livers of fetal, newborn, and adult rats has been studied. The results indicate that the enzyme system develops shortly after birth. Activity of the enzyme is negligible in livers of fetal rats near term and rats less than 24 hours old; it approaches the adult level in livers of animals only a few days old. The virtually inactive preparations obtained from livers of fetal and newborn animals could not be activated by adding increasing amounts of pyridine nucleotide to the reaction mixtures in either oxidized or reduced form. The activity of soluble fractions of adult liver was partially lost during short dialysis; the initially low activity of preparations of livers from fetal and newborn animals was particularly sensitive to this procedure.

† F. T. Kenney, *et al.*, in preparation.

1. Jervis, G. A., *J. Biol. Chem.*, 1947, v169, 651.

2. Udenfriend, S., and Bessman, S. P., *ibid.*, 1953, v203, 961.

3. Jervis, G. A., PROC. SOC. EXP. BIOL. AND MED., 1953, v82, 514.

4. Wallace, H. W., Moldave, K., and Meister, A., *ibid.*, 1957, v94, 632.

5. Mitoma, C., Auld, R. M., and Udenfriend, S., *ibid.*, 1957, v94, 634.

6. Kretchmer, N., and Etzwiler, D. D., to be published in *Pediatrics*, 1957.

7. Nemeth, A. M., *J. Biol. Chem.*, 1954, v208, 773.

8. Kretchmer, N., and McNamara, H., *J. Clin. Invest.*, 1956, v35, 1089.

9. Udenfriend, S., and Cooper, J. R., *J. Biol. Chem.*, 1952, v196, 227.

10. Ma, T. S., and Zuazaga, G., *Indust. and Engin. Chem. Anal. Ed.*, 1952, v14, 280.

11. Kaufman, S., *Fed. Proc.*, 1957, v16, 871.

12. ———, *J. Biol. Chem.*, 1957, v226, 511.

13. Udenfriend, S., and Mitoma, C., *in Amino Acid Metabolism*, W. D. McElroy and B. Glass, eds. 1955, Johns Hopkins Press, Baltimore.

14. Mitoma, C., *Arch. Biochem. and Biophys.*, 1956, v60, 476.

Received August 1, 1957. P.S.E.B.M., 1957, v96.

Role of Brown Fat in Pathogenesis of Rabies in Insectivorous Bats (*Tadarida b. mexicana*)* (23507)

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At least 2 human rabies deaths are believed to have resulted from exposure to bats in this country. The first was reported by Sulkin and Greve(1) and concerns a woman who died in October, 1951. The second, a member of the Entomology Section of the Texas State Department of Health, occurred in January, 1956(2). These cases, together with isolation of the rabies virus from a number of species of bats in widely scattered areas of the United States has resulted in a reappraisal of the public health significance of these animals(3,4). The initial isolation in 1953 of the rabies virus from bats in Florida(5) and the almost simultaneous isolation of the virus from other bat species by other investigators (6,7) stimulated teams of mammalogists and virologists to investigate the ecology of rabies in different geographic areas. The accumulating literature indicates that most of the studies thus far conducted have been concerned with determining which bat species are naturally infected. Only a limited number of studies have dealt with the progress of the disease in experimentally infected bats(8-11).

Recent studies by Shwartzman(12,13) demonstrating lipotropism in cortisone-treated hamsters infected with poliovirus together with previous studies concerned with the effects of environmental temperature and hormonal factors on experimental virus infections (14,15) suggested an approach to the study of the role of the bat in the biological life cycle of the rabies virus. Aronson and Shwartzman (16) demonstrated reproducible histologic lesions and high infective virus titers in brown (embryonal) fat in cortisone-treated hamsters infected with poliovirus, indicating marked lipotropism in this infection. These observations have been confirmed in part by Bodian (17) who showed high concentrations of virus in brown fat in chimpanzees after the viremic phase of the infection, and suggested that the brown fat might be regarded as a "target organ" or secondary site of poliovirus multiplication. Invasion of brown fat has also been observed in suckling mice infected with certain strains of Coxsackie virus(18,19,20) but no other examples of lipotropism in viral infection have come to our attention. The known effects of environmental temperature and hormonal factors on experimental viral infections(14,15) together with the observed sensitivity of brown fat to these and other stress mechanisms(21) suggest that this tissue might in some way be involved in the persistence of the rabies virus in symptomless carriers. Furthermore, the susceptibility of this tissue to certain virus infections is so great that it seems to persist when most other organs and tissues have become refractory (18).

The purpose of the present study has been to examine the pathogenetic sequence in experimentally infected insectivorous bats with the view to learning something of the possible mechanism by which these animals may serve as persisting reservoirs for the virus in nature. An effort has been made to study the pathogenesis of rabies in this host with the view to finding anatomical sites of viral multiplication. The results to be reported suggest that the brown (hibernating) fat provides a depot for storage of the virus. Furthermore, these studies provide another example of viral lipotropism.

Methods. Because of the tremendous populations of Mexican free-tailed bats (*T. braziliensis mexicana*) in the southwestern part of the U.S. and since a large proportion of the rabies virus isolations were made from this species, this bat was used in these studies.†

* Supported by grant from Caruth Fn., Dallas, Texas.

† We should like to acknowledge free access to the Blowout Cave (Blanco County, Texas) granted by Mr. Gould Davis, rancher and owner, without whose cooperation this study could not have been completed. We also wish to acknowledge the assistance of Mr. William Hanszen, rancher, who helped in netting bats.

Technics for the safe laboratory care and successful maintenance of infected experimental populations for long periods of time have been described elsewhere(22). The rabies street virus used in the present study, and designated the Thompson strain, was isolated in albino Swiss mice (CFW) from a human brain and had been through two intracerebral passages in white mice. The LD_{50} titer of the stock virus suspension for 3-week-old white Swiss mice inoculated intracerebrally was $10^{-4.8}$. Bats were inoculated intramuscularly into the heavy muscle over the breast. The inoculum consisted of approximately 8000 mouse intracerebral LD_{50} contained in 0.1 ml. The Mexican free-tailed bats (*Tadarida b. mexicana*) were netted on the day the experiment was initiated and were chilled to facilitate handling during inoculations and distribution into cages. The inoculated animals were maintained at 29°C ± 2 (relative humidity 65%) and were fed daily during the observation period of 3 months. All bats were observed daily for symptoms of rabies. Those which died during the first week were discarded. The selective affinity of various tissues of the bat for rabies virus was shown by viral assay in 3 to 4-week-old white Swiss mice (CFW). Tissues were preserved in a mechanical refrigerator at –40°C until ready to be tested. For qualitative assay of brain, salivary gland, and interscapular brown fat 3-5 mice were inoculated intracerebrally with each specimen (10% brain, 2% salivary gland, and 2-5% brown fat) and observed for a 30-day period before being discarded as negative. In the quantitative assays serial 10-fold dilutions of the respective tissues were inoculated intracerebrally into groups of 5 mice each and the LD_{50} titer was established in each instance by the method of Reed and Muench(23). Since Negri bodies are not always demonstrable in tissues of bats proven to contain virus by mouse inoculation(8,11) histologic examination of the various tissues was not made routinely.

Results. Of the 163 Mexican free-tailed bats inoculated intramuscularly with rabies virus, 26 died during the first 7 days after the virus inoculation and have been excluded from the analysis.‡ Qualitative assays for virus showed evidence of rabies infection during the observation period of 3 months in 32 or 23.3% of the remaining 137 bats. Virus was detectable in the brain in 28 (20.5%) salivary glands in 10 (7.5%), and in the interscapular brown fat in 11 (8.0%). Table I summarizes the data on 11 bats in which the brown (hibernating) fat contained detectable or significant amounts of rabies virus. It appears from these data that the virus is most widely distributed in this host between the 20th and 30th days following intramuscular inoculation. In addition there seems to be more active viral proliferation during this period of the infection. In 4 instances rabies virus was detectable in the brown fat and not in the brain or salivary gland. These animals, however, were sacrificed between the 32nd and 75th days after the virus inoculation. The remaining 21 bats which showed evidence of rabies infection all contained virus in the brain with occasional demonstration of virus in the salivary glands. A small percentage (<2%) of the bats used in these experiments may have been naturally infected with rabies virus.

TABLE I. Demonstration of Rabies Virus in Interscapular Brown Fat and Other Tissues of Bats (*Tadarida b. mexicana*) Inoculated Intramuscularly and Maintained at 29°C.

Duration of infection*	Rabies virus demonstrated in		
	Brain	Salivary gland	Brown fat
22	+ (2.43)†	+ (2.33)	+ (1.66)
22	+ (2.75)	—	+
22	+ (3.5)	+ (2.75)	+ (3.0)
22	+ (2.5)	—	+ (1.5)
25	+ (3.0)	—	+ (1.5)
26	+	—	+
32	—	—	+ (2.5)
32	—	—	+
42	+	+ (2.75)	+
75	—	—	+ (1.5)
75	—	—	+ (1.5)

* Indicates day after intramusc. inoc. (0.1 ml) bat found dead or sacrificed. Virus inoculum ± 8000 mouse i.c. LD_{50}.

† Figure in parentheses indicates neg. log LD_{50} i.c. titer for mice.

In the few instances in which symptoms in-

‡ Studies(22) concerning the laboratory care and maintenance of this bat species have indicated that those which survive 7 or 8 days in captivity can usually be kept for several months or longer.

cluding irritability and aggressiveness occurred prior to death it was clear that the incubation period in bats following intramuscular inoculation of virus was considerably longer than in mice inoculated intracerebrally, thus confirming previous reports with other species of bats and different virus strains(8, 11). A few Mexican free-tailed bats inoculated intracerebrally with the Thompson strain of rabies virus used in these experiments also showed longer incubation periods than mice inoculated by the same route.

Whether or not animals developed symptoms of rabies and recovered could not be clearly established in these experiments because of the difficulties involved in handling these animals.

Discussion. In the present studies only 23% of Mexican free-tailed bats showed evidence of infection following intramuscular inoculation of rabies virus, and only a small proportion of these developed symptoms of rabies. This is not surprising since Enright and his associates(10) had shown that none of a limited number of the same species of bats inoculated intramuscularly with a strain of rabies virus originally isolated from *Tadarida* developed rabies symptoms. Whether these animals became infected was not established. Burns and his coworkers(8) have also shown that some *Tadarida b. mexicana* fail to develop symptoms of rabies; virus could be demonstrated in brain and salivary gland even though the animals showed no clinical evidence of illness. In earlier studies with vampire bats, Johnson(24) reported that in experimentally infected bats the incubation periods ranged from 7 to 171 days and some animals failed to develop the disease. Failure to infect a larger proportion of the bats by the intramuscular route of inoculation may have been due to the presence of neutralizing antibodies in many of these animals(8).§

The studies described in this report suggest that the rabies virus is capable of proliferation in the interscapular brown fat of the Mexican free-tailed bat following intramuscular inoculation. The brown fat may constitute a depot or "target organ" in which a hardy agent like the rabies virus is likely to persist for long periods of time. Virus has been demonstrated in this tissue in bats showing no overt signs of the disease 75 days after intramuscular inoculation of the virus. Preliminary studies to be reported later have already indicated that gravid bats‖ are more susceptible than nongravid animals, thus serving as a possible mechanism for activation and perpetuation of the agent in this spring-breeding species. While the function of brown fat is not clearly established, there is evidence that it suppresses metabolism during winter sleep (25). Since brown fat is seasonally variable in amount and most abundant during the winter months, it is likely that the decreased metabolic rate during periods of cold weather may be concerned with the latent period of the rabies virus in bats. At such times one would expect less active viral proliferation. Whether virus can be demonstrated in brown fat of bats under natural circumstances remains to be determined.

Studies designed to determine the route by which the brown fat is invaded by the rabies virus are in progress. It is hoped that temporal studies will establish the mode of progression of the virus following peripheral inoculation.

These studies in addition to including the rabies virus among those (poliovirus and certain Coxsackie viruses) which exhibit lipotropism suggest that this may prove to be a characteristic feature of infection with "hardy" viruses.

Summary. 1. Studies on the progression of peripherally inoculated rabies virus in Mexican free-tailed bats (*Tadarida b. mexicana*) suggest that the brown (hibernating) fat plays a significant role in the pathogenesis of rabies in this animal. 2. Brown fat may serve as a depot for the storage of virus in symptomless carriers of the rabies virus. 3. These studies provide another example of selective viral lipo-

§ It should be noted, however, that Schneider and his colleagues(4) failed to demonstrate neutralizing antibodies in another species of *Tadarida* (*T. cynocephala*) in another geographic area.

‖ Rabies virus has been demonstrated in the brain tissue of 2 infant bats suckling on an experimentally infected bat.

tropism.

1. Sulkin, S. E., and Greve, M. J., *Texas State J. Med.,* 1954, v50, 620.

2. Irons, J. V., Eads, R. B., Grimes, J. E., and Conklin, A., *Texas Rep. Biol. and Med.,* 1957, v15, 292.

3. Enright, J. B., *Ann. Rev. Microbiol.,* 1956, v10, 369.

4. Schneider, N. J., Scatterday, J. E., Lewis, A. L., Jennings, W. L., Venters, H. D., and Hardy, A. V., *Am. J. Public Health,* 1957, v47, 983.

5. Venters, H. D., Hoffert, W. R., Scatterday, J. E., and Hardy, A. V., *ibid.,* 1954, v44, 182.

6. Sullivan, T. D., Grimes, J. E., Eads, R. B., Menzies, G. C., and Irons, J. V., *Public Health Repts.,* 1954, v69, 766.

7. Burns, K. F., and Farinacci, C. F., *J. Inf. Dis.,* 1955, v97, 211.

8. Burns, K. F., Farinacci, C. F., and Murnane, T. G., *Am. J. Public Health,* 1956, v46, 1089.

9. Reagan, R. L., Delaha, E. C., Rumbaugh, H. L., and Brueckner, A. L., *Cornell Vet.,* 1955, v45, 153.

10. Enright, J. B., Sadler, W. W., Moulton, J. E., and Constantine, D., PROC. SOC. EXP. BIOL. AND MED., 1955, v89, 94.

11. Stamm, D. D., Kissling, R. E., and Edison, M. E., *J. Inf. Dis.,* 1956, v98, 10.

12. Shwartzman, G., PROC. SOC. EXP. BIOL. AND MED., 1950, v75, 835.

13. ———, *ibid.,* 1952, v79, 573.

14. Sulkin, S. E., *J. Immunol.,* 1945, v51, 291.

15. Sulkin, S. E., Wallis, C., and Donaldson, P., *J. Inf. Dis.,* 1952, v91, 290.

16. Aronson, S. M., and Shwartzman, G., *Am. J. Path.,* 1956, v32, 315.

17. Bodian, D., *Am. J. Hyg.,* 1956, v64, 181.

18. Pappenheimer, A. M., Daniels, J. B., Cheever, F. S., and Weller, T. H., *J. Exp. Med.,* 1950, v92, 169.

19. Dalldorf, G., *Bull. N. Y. Acad. Med.,* 1950, v26, 329.

20. Godman, G. C., Bunting, H., and Melnick, J. L., *Am. J. Path.,* 1952, v28, 223.

21. Shwartzman, G., Aronson, S. M., Teoduro, C. V., Adler, M., and Jahiel, R., *Ann. N. Y. Acad. Med.,* 1955, v61, 869.

22. Krutzsch, P. H., and Sulkin, S. E., *J. Mamm.*

23. Reed, L. J., and Muench, H., *Am. J. Hyg.,* 1938, v27, 493.

24. Johnson, H. N., *Oxford Univ. Press,* 1943, v5, 599.

25. Wells, H. G., *J. Am. Med. Assn.,* 1940, v14, 2177.

Received August 1, 1957. P.S.E.B.M., 1957, v96.

Inhibitory Influence of a Normally Occurring Pyrimidine Precursor upon Methylcholanthrene Carcinogenesis.* (23508)

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In studies of the mechanism of action of urethane in initiating pulmonary adenomas in the lungs of mice, orotic acid, a naturally occurring pyrimidine(1) known to play a large part in nucleic acid synthesis(2) was found to be one of the most potent inhibitors of the sequential metabolic processes originally set off by the carcinogen(3). The following experiments were carried out to determine whether it might influence the carcinogenic effect of methylcholanthrene, a polynuclear hydrocarbon. The fact that polynuclear hydrocarbons exert their effect over a long period of time necessitated, however, the use of a different technic from that carried out in the studies of the short acting carcinogens (3,4).

Materials and method. Mice of the Swiss and "C" strains, raised in this laboratory and procured originally from the stock of the Rockefeller Institute for Medical Research, New York City, and mice of the "A" strain procured from Bar Harbor, Maine, were used. The Swiss and "A" strain animals develop large numbers of pulmonary tumors following administration of a variety of carcinogens. In the "C" strain the tumors are much less numerous. In each experiment the animals used were of the same sex and matched as to age and weight. They were all kept on a stock diet (Laboratory Mouse Chow, made by Dietrich and Gambrill, Frederick, Md.) This was

* Supported by grant from Natl. Cancer Inst., N.I.H., U.S.P.H.S.

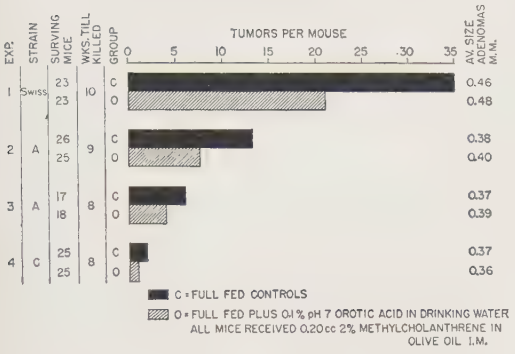

CHART 1. Inhibition of methylcholanthrene carcinogenesis by increasing dietary orotic acid.

supplemented twice weekly with whole wheat bread and milk. All mice were injected with 0.2 cc of 2% methylcholanthrene (Eastman Kodak Laboratories, Rochester, N. Y.) in olive oil intramuscularly in the right thigh. Two to 5 days prior to the methylcholanthrene injection half the animals were given 0.1% pH 7 orotic acid (Nutritional Biochemicals Corp., Cleveland, O.) in tap water as their normal water supply. The control animals received only tap water. The mice receiving orotic acid in Exp. 1 and 4 received it for the duration of the experiment. Those of Exp. 2 and 3 were given tap water for the last 4 weeks of the experiment. There was no difference in the relative fluid intake of experimental and control groups. The weights and general condition of the animals were followed closely because these factors significantly influence the number of tumors occurring following exposure to carcinogens(5). Animals which developed intercurrent disease or deviated in weight from the mean as much as 2 g were discarded. The mice were killed by decapitation in each experiment at the interval indicated in the Chart and their lungs examined for tumors. Only tumors on the surface of the lungs were measured and counted, and among these only those one-third mm in diameter and over figured in the results.

Results. In the accompanying chart it is evident that the mice receiving supplementary orotic acid developed significantly fewer tumors than did the controls. Since the weight curves of the compared groups were held constant by discarding deviates they are not included in this report. The constancy of the weights of the 2 groups under these experimental conditions is indicated by the fact that the number of surviving animals in the groups is essentially equal. This together with the equality in average size of adenomas from group to compared group makes it clear that non-specific growth effects could not account for the reduction in the number of tumors in the animals receiving orotic acid and further that the inhibitory influence of orotate is upon the initiation and not the subsequent growth of the tumors(6). The tumors produced in the lungs were all of the general class variously categorized as pulmonary adenomas or adenocarcinomas. The infrequency of the appearance of sarcomas at the site of injection of the carcinogen in these experiments does not permit the use of this feature in making a comparison of the response of the two groups of animals.

Summary. The carcinogenic influence of methylcholanthrene may be considerably reduced by increasing the intake of a normal dietary constituent, orotic acid, a substance well known to be of great importance to the synthesis of nucleic acid pyrimidines. The absence of influence of this substance upon the condition of the mice or the size of the tumors indicates that it is acting at the level of initiation of the pulmonary tumors rather than upon their subsequent growth. This fact suggests that the metabolic mechanisms through which methylcholanthrene initiates neoplastic change is, like urethane(3), closely related to the synthesis of nucleic acid.

1. Bascaro, G., and Belloni, E., *Chem. Sentr. Ann. Soc. chim. Milano,* 1905, v11, No. 1.

2. Reichard, P., and Lagerkvist, U., *Acta Chem. Scand.,* 1953, v7, 1207.

3. Rogers, S., *J. Exp. Med.,* 1957, v105, 279.

4. ———, *J. Nat. Cancer Inst.,* 1955, v15, 1001, 1379, 1675.

5. Tannenbaum, A., *Am. J. Cancer,* 1940, v38, 335.

6. MacKenzie, I., and Rous, P., *J. Exp. Med.,* 1941, v73, 391.

Received August 6, 1957. P.S.E.B.M., 1957, v96.

Potentiation of Polymyxin B Toxicity by ACTH.*† (23509)

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The degree of polymyxin B toxicity has been found to be a function of the concentration of heparin in the tissues and of the dose of polymyxin employed(1). The basic polypeptide, polymyxin B, and the acidic mucopolysaccharide, heparin, form a complex *in vitro* which does not readily dissociate *in vivo*. This complex can be sequestered by fibroblasts and deposited as intracytoplasmic metachromatic granules (quasi-mast cells)(2). Acute polymyxin toxicity thus appears to be due to the amount of polymyxin in excess of that amount fixed by endogenous acidic mucopolysaccharides of the tissues. It has been postulated that heparin can act in the tissues as an ion exchanger(3). The enhanced polymyxin B tolerance of heparin-treated mice led to the assumption that the natural capacity for polymyxin tolerance is dependent on an ion exchange function of the tissues. The ability of an animal to tolerate polymyxin is represented as a function of the capacity of acidic macromolecules in its tissues to exchange naturally bound substances for polymyxin. The release of histamine *in vivo* by polymyxin(4) and the degranulating effect of polymyxin on the heparin- and histamine-containing mast cells of connective tissue(5) would support this assumption. As is well known, the capacity of an ion exchanger is a quantitative measure of its ability to exchange one substance for another and, during the course of its operation, this capacity becomes progressively smaller. In the following study the possibility has been examined that a naturally occurring substance which can form a complex with heparin may decrease the capacity of the tissues to fix polymyxin and thereby relatively decrease the capacity of polymyxin tolerance in the animal. The results obtained with the basic protein, adrenocorticotrophic hormone (ACTH) are in accord with this idea and indicate that ACTH can non-specifically potentiate polymyxin toxicity in a dose-response manner.

Materials and methods. Complex formation. The formation of heparin complexes with various drugs was determined as follows: The system consists of 120 μg of the dye, toluidine blue (G. Grubler & Co.), in complex with 50 μg of heparin (117 u/mg, The Upjohn Co.)‡ in a series of tubes. Graded amounts of the test substances were added to make a final volume of 6 cc, all substances having been dissolved in deionized water. Toluidine blue and heparin rapidly form a complex which has an optimal absorption at 540 mμ in contrast to the optimal absorption of "free" toluidine blue at 630 mμ(6). The dissociation of the toluidine blue-heparin complex is enhanced in the presence of any of a number of basic substances which have an affinity for heparin. The dissociated dye is freely dialyzable, and, at optimal proportions, the newly formed drug-heparin complex forms a definite precipitate. The amount of free dye was determined with a Coleman Jr. Spectrophotometer at a wave length of 630 mμ and the results plotted against the respective amounts of basic substance used. The relative affinities of various substances for heparin were then compared.

Toxicity studies. All materials for injection were prepared in isotonic, pyrogen-free, sterile saline and were injected by the intravenous route in a volume of 0.25 ml. Polymyxin B (Aerosporin)‡ dose was based on the biological equivalent of the 50 mg polymyxin B standard (500,000 units of activity). CBA mice, 2 to 3 months of age and ranging in weight from 20 to 22 g, were challenged with a standard *sublethal* dose of polymyxin (3.5 μg/g body weight) or mixtures of this stand-

* Research supported by grant from Department of the Army.

† The authors wish to acknowledge the technical assistance of Mr. Blair Beck.

‡ The authors wish to express their appreciation to Dr. L. L. Coleman of The Upjohn Co., Dr. S. T. Bloomfield of Burroughs Wellcome & Co. and Dr. James B. Lesh of Armour Laboratories for their generous gifts of heparin, polymyxin B and ACTH, STH, and TSH and chymotrypsin, respectively.

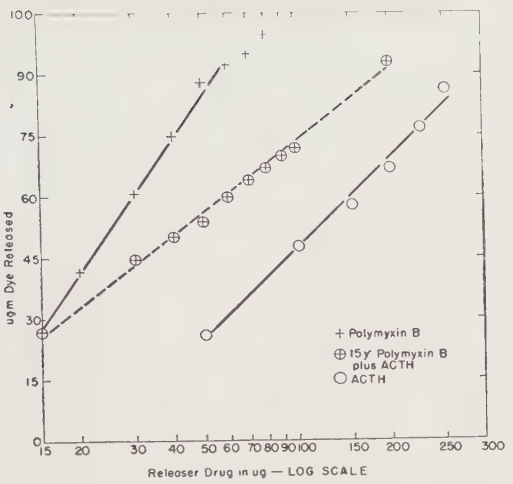

FIG. 1. Comparison of dye release activities of 1) graded amounts of polymyxin B, 2) a constant amount of polymyxin B (15 μg) with graded amounts of ACTH and 3) graded amounts of ACTH. Note additive effect of ACTH on release of toluidine blue from heparin complex by polymyxin B.

ard polymyxin dose with a given amount of one of the test substances. Control mice were given the individual drugs in the same amounts and manner. Toxicity was determined on the basis of number of animals surviving for 24 hours following challenge. However, under the conditions of this study deaths usually occurred within 5 minutes after injection. The substances tested for their heparin affinity as well as for their effect on polymyxin toxicity were: ACTH (ACTHAR, H.P., lyophilized), STH (Somatotropin, lyophilized), TSH (Thyrotropar, lyophilized) and Chymotrypsin from Armour and Co.,‡ clupein sulfate (K and K Laboratories) and neomycin sulfate (665 μg/mg, Nutritional Biochemicals Corp.) and toluidine blue.

Results. In vitro. The toluidine blue-heparin complex was used as an *in vitro* model system to simulate a readily dissociable state of acidic macromolecules in the tissue. The interaction of polymyxin with this heparin complex as evidenced by the release of toluidine blue is shown in Fig. 1. Since the dissociated dye is freely dialyzable and, at optimal proportions, the polymyxin-heparin complex is visible as a definite precipitate, the polymyxin can be designated in this system either as a "releaser" of toluidine blue or as a "binder" of heparin. The concomitant formation of a heparin complex with the polymyxin releaser is also indicated by the markedly reduced toxicity of polymyxin when complexed with heparin either *in vitro*(7) or *in vivo*(2). Therefore, the release of dye is considered as indirect evidence of the formation of a complex between the "releaser" and heparin.

As indicated in Fig. 1, the amount of dye released is proportional to the log concentration of polymyxin within the measured range of 15 to 80 μg polymyxin per 6 cc of the test system. It is also shown in Fig. 1 that with a constant amount (15 μg) of polymyxin and graded amounts (15-100 μg) of ACTH, the total amount of dye released/μg of polymyxin is progressively greater but proportional only to the ACTH concentration. Thus the release of dye by polymyxin is of a non-specific nature. Actually, as shown in Fig. 1, ACTH also has a dye releasing activity which is consistent with an ability to bind with heparin (8). Therefore ACTH has an *additive* effect on this *in vitro* dye releasing activity of polymyxin. Since the biological effects of polymyxin and ACTH are apparently so dissimilar, this additive effect of ACTH would be manifested *in vivo* as a *potentiation* of a polymyxin effect in the tissues (*i.e.*, toxicity). In other words, ACTH could compete with polymyxin for binding with acidic mucopolysaccharides in the tissues and in this manner serve to deplete the total number of active sites otherwise available for the fixation of polymyxin. The net effect of this competition would be to increase the amount of polymyxin free to act at other sites in the tissues and would be equivalent to the effects of larger doses of polymyxin.

In vivo. To evaluate the possibility that ACTH could competitively interact with polymyxin "binding sites" in the tissue, mixtures containing a standard *sublethal* dose (3.5 μg/g) of polymyxin and graded amounts (0-37.5 μg/g) of ACTH were used as intravenous challenges for a number of groups of mice. The experimental groups consisted of 10 animals each. The intravenous route was used for challenge since this is the most sensitive method for eliciting the toxic effects of polymyxin.

In accord with the assumption made, in-

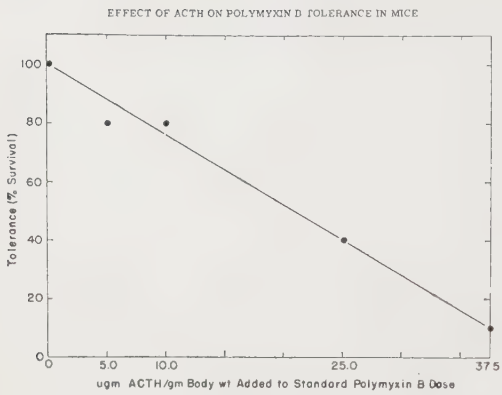

FIG. 2. Effect of ACTH on polymyxin B tolerance of 11 weeks old CBA mice (10 mice/group). Animals were injected intravenously with a standard *sublethal* dose of polymyxin B containing graded amounts of ACTH (abscissa). Note that tolerance decreases as ACTH dose increases.

creasing doses of ACTH should progressively decrease heparin-like sites in the tissues otherwise available for interaction with a given amount of polymyxin. Concomitantly, there should be an increase in the relative amount of polymyxin available to interact with more vital sites or activities in the tissues. The postulated ACTH effect should therefore be measurable in terms of a decreased polymyxin tolerance relative to the ACTH dose. In Fig. 2, the per cent survival has been plotted against the respective dose of ACTH (μg/g) contained in the polymyxin-ACTH mixture. It can be noted that mice receiving the control polymyxin dose had a 100% survival. In related experiments a total of more than 35 mice have received this standard polymyxin dose without lethal effects (see Table I). However, those animals given 5 to 10 μg ACTH/g with the polymyxin dose had only an 80% survival; 25 μg ACTH/g: 40% survival; and 37.5 μg ACTH/g: 10% survival. Larger amounts of ACTH (50 μg/g) have been found to decrease the survival to 0%. Control doses of ACTH alone had no obvious toxic effects (see Table I). These results indicate that although the interaction of ACTH with tissue constituents does not of itself induce lethal toxicity, this interaction can reduce tolerance for polymyxin by interfering with tissue mechanisms necessary for dealing with this latter drug. The net effect is a potentiation of polymyxin toxicity by ACTH.

TABLE I. Comparison of Various Drugs for Effect on Polymyxin Tolerance and for Dye Release Activity.

Group	Treatment and survival ratios — Drug doses (μg/g) 25	12.5	6.25	50% dye release activity (μg)
		ACTH		150
Control	10/10			
Pmx*	6/15			
		STH		Ineffective
Control	10/10			
Pmx*	"			
		TSH		"
Control	"			
Pmx*	"			
		Chymotrypsin		"
Control	"			
Pmx*	"			
		Clupein		34.5
Control	"			
Pmx*	1/10	3/10	10/10	
		Neomycin		20
Control	0/10	10/10		
Pmx*		0/10	7/10	
		Toluidine blue		(60)
Control	10/10			
Pmx*	0/10	5/10	8/10	
		Polymyxin		30
Control			0/10	
Pmx*			"	
		Saline		—
Control				
Pmx*	35/35			

* Challenge consists of respective drug dose as a mixture containing a sublethal dose (3.5 μg/g) of polymyxin B.

Specificity. The importance of ACTH as a hormone made it imperative that further experiments be performed to determine the specificity involved in the ACTH effect on tolerance to polymyxin. Accordingly, a number of other substances were selected for comparison with ACTH. Groups of mice were treated as before and the standard polymyxin dose was given as a mixture with different amounts (25.0, 12.5 or 6.25 μg/g) of the test substances and their effects on the survival ratios were noted. In an associated study, the effect of each of the test drugs on the dissociation of the toluidine blue-heparin complex was also determined. The amount of test substance required to release 50% (60 μg) of the dye from its heparin complex was designated as the *50% releaser activity* of the drug.

A substance was arbitrarily considered as ineffective if more than 1 mg of it was required for 50% releaser activity. Comparisons of the various drugs on the basis of their effects on survival ratio and their releaser activities can be observed in Table I.

A comparison of ACTH with STH and TSH, respectively, indicated that ACTH was the only one of these protein hormones from the adenohypophysis which had a demonstrable effect on survival ratios or was an effective dye releaser. Chymotrypsin, another protein with biological activity, had no significant effect on survival or dye release. However, clupein, a basic protein derived from a nucleoprotamine, proved to have marked effect on survival ratios and also proved to be an effective releaser substance. The non-specificity of the ACTH effect on polymyxin tolerance can thus be seen and it would appear that this effect is related to the basic protein character of ACTH. Moreover, neomycin, a basic polypeptide-like antibiotic substance, also had a definite effect on survival ratios at dose levels which were not lethal to control mice. This substance also had a pronounced dye releaser activity.

Toluidine blue, a basic thiazine dye with a well known affinity for heparin either *in vitro* (6) or *in vivo*(9) also potentiated the toxicity of polymyxin. This observation gives further support to the idea that the potentiation of polymyxin toxicity by these basic substances is a result of their competition with polymyxin for certain non-specific bindings with acidic polyelectrolytes of the tissues. Furthermore, it is obvious from the table that polymyxin itself is the most active potentiator of polymyxin toxicity and that, like all other substances found to potentiate polymyxin toxicity, it also has an effective dye release activity.

Discussion. A potential affinity of ACTH for various acidic constituents of the tissues has been discussed(10) with emphasis on the significant relationship between the basic character of ACTH and the acidic nature of the basophilic cells of the adenohypophysis. Reports that ACTH complexes with heparin *in vitro*(8) and possibly *in vivo*(11) give further support to this idea. Similarly, the basic character of polymyxin B, and its affinity for phosphorylated constituents of the bacterial cell(12) as well as its formation of complexes with heparin either *in vitro* or *in vivo*(2) indicate that it also has a potential for interacting with various acidic constituents of the tissues. Obviously, such interactions would be partially non-specific and this would imply that ACTH and polymyxin B could non-specifically compete for acidic binding sites in the tissues. This assumption is supported by the observations in the present study that both ACTH and polymyxin enhance the dissociation of a toluidine blue-heparin complex (*i.e.*, heparin binding) *in vitro*, that they have an additive effect on this dissociation, and that ACTH potentiates polymyxin toxicity *in vivo*. These observations as well as those by other workers indicate that ACTH can participate in biological activities of an extra-adrenocortical nature. For instance, ACTH has been found to inhibit the conjugaton of cortisol by the liver(13). With regard to the interaction of ACTH with other basic substances, recent studies by other laboratories have indicated that the basic polypeptide, pitressin, has a measurable and perhaps specific ACTH releaser activity *in vivo*(14), that the basic polypeptide, glucagon, can exert a sparing effect on demonstrable ACTH in adenohypophyseal minces(15) and conversely, that alpha corticotrophin, etc., can potentiate the activity of otherwise ineffective amounts of glucagon *in vitro*(16). The effect of ACTH on polymyxin toxicity reported here would appear to be only one of a number of examples in which a biological activity of a given basic substance is markedly enhanced by the presence of another. In the present instance, the effects would not require the direct intervention of specific enzymatic processes for their demonstration and could be most easily explained as the result of a competition between the basic substances for the same acidic sites in the tissues. Thus, with a limited number of acidic sites capable of binding polymyxin, the amount of polymyxin bound would be relative to the concentration of other substances capable of competing with polymyxin for these sites. Since polymyxin toxicity is dose dependent and the depletion of non-specific binding sites in the

tissues would increase the relative amount of polymyxin available for interaction with more vital sites or activities in the tissues, ACTH enhances the toxicity of polymyxin B. In this manner, an otherwise sublethal amount of polymyxin becomes a fatal dose.

The reduction of polymyxin tolerance by clupein, neomycin or toluidine blue (which are structurally unrelated to ACTH, polymyxin or to each other) indicates the non-specificity involved in the effects of such substances on the *capacity* of the tissues to tolerate polymyxin. The effects of these substances on polymyxin tolerance can be correlated by their common affinity for heparin *in vitro*. Heparin as well as other acidic substances such as chondroitin sulfate B or any of a variety of chemically sulfonated macromolecules have been found to enhance the tolerance of these animals for polymyxin(1,7). Theoretically, polymyxin tolerance in the tissues can be described as a function of endogenous heparin. The term "heparin" is used here in a generic sense to include those tissue substances capable of complexing with polymyxin such as other sulfonated polysaccharides. It has been proposed that the mechanism in the tissues whereby heparin interacts with polymyxin, etc., is related to the capacity of heparin to serve as an ion exchanger (*i.e.*, a readily dissociable state of heparin)(3). In this respect, heparin can become relatively unavailable for interaction with polymyxin if the capacity of this exchanger has been reduced as a consequence of its interaction with other substances which also have a strong affinity for this tissue mucopolysaccharide. The linear dose effect of ACTH on polymyxin tolerance can readily be explained on this basis. Therefore, the capacity to tolerate a given amount of polymyxin can be measurably altered in one direction or another (tolerance versus toxicity) by certain naturally occurring substances in the tissues (heparin versus ACTH).

It may be held unlikely that a sufficient amount of endogenous ACTH is present following stress to produce toxicity according to the point of view given here. Basic substances acting as stressors and the accumulated basic polypeptides released from injured cells could all compete for sulfonated macromolecules and this summation of complexes could produce stress by exceeding the ion exchange capacity of the tissue. This possibility has been explored and will be reported later.

Summary. An *in vitro* technic has been used to detect the interaction of various basic substances (ACTH, polymyxin, clupein and neomycin) with heparin, an acidic mucopolysaccharide of the tissues. It was observed that substances effectively interacting with heparin in this *in vitro* system markedly potentiated the toxicity of polymyxin B *in vivo*. It has been suggested that polymyxin tolerance is dependent on an ion exchange function of heparin-like constituents of the tissues and that the capacity for tolerance can be reduced by substances which have a strong affinity for these heparin-like constituents.

1. Higginbotham, R. D., and Carter, P. B., *Antibiotics and Chemother.*, 1957, v7, 527.

2. Higginbotham, R. D., and Dougherty, T. F., *Res. Bull.* No. 2, Fall, 1956, v2, p27.

3. ———, *Fed. Proc.*, 1957, v16, March, 58.

4. Bushby, S. R. M., and Green, A. F., *J. Pharmacol. Chemother.*, 1955, v10, 215.

5. Norton, S., and de Beer, E. J., *Arch. Internat. Pharmacol. Therap.*, 1955, v10, 352.

6. MacIntosh, F. C., *Biochem. J.*, 1941, v35, 776.

7. Unpublished results.

8. Köhler, J., Loll, H., and Schröer, H., *Klin. Wschr.*, 1953, v31, 616.

9. Allen, J. G., and Jacobson, L. O., *Science*, 1947, v105, 388.

10. Pernis, B., Esselier, A. J., and Morandi, L., *Klin. Wschr.*, 1954, v32, 204.

11. Weissbäcker, L., and Schröter, A., *Acta Endocrinol.*, 1954, v15, 66.

12. Newton, B. A., *Bact. Rev.*, 1956, v20, 14.

13. Dougherty, T. F., and Berliner, D., 1957, in preparation.

14. McCann, S. M., *Endocrinology*, 1957, v60, 664.

15. Barrett, A. M., Acoba, K., and Sayers, G., *The Endocrine Soc.* (abst.) 1957, p30.

16. Tyberghein, J. M., Tomizawa, H. H., and Williams, R. H., *J. Biol. Chem.*, 1956, v22, 945.

Received August 12, 1957. P.S.E.B.M., 1957, v96.

Effect of Ethylenediaminetetraacetate on Lipide Peroxide Formation and Succinoxidase Inactivation by Ultraviolet Light.* (23510)

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Exposure of cells, mitochondria and other biological material to ultraviolet light catalyzes the formation of lipide peroxides. The observed inhibition of biological activity (cell division, mitochondrial enzymes) upon irradiation is proportional to the amount of lipide peroxides formed. Similar effects can be produced by lipide peroxides when they are added to the various biological systems(1-5). This suggests that these peroxides may act as mediators of the effects produced by irradiation. If this is the case, then substances which interfere with lipide peroxide formation should also inhibit the biological effects of ultraviolet irradiation. To test this hypothesis, experiments were carried out with ethylenediaminetetraacetate (EDTA) which as the following results indicate, prevents peroxide formation in liver homogenates and mitochondria exposed to ultraviolet light. The results show that it is possible to protect the succinoxidase in mitochondria from inhibition by ultraviolet light if EDTA is present and that the protection is proportional to the inhibition of peroxide formation. Unlike its action in homogenates and mitochondria EDTA does not prevent peroxide formation in pure methyl linolenate emulsions similarly exposed to ultraviolet light.

Materials and methods. Homogenates were prepared from rat liver in cold isotonic KCl buffered at pH 7.0 with Na-K phosphate buffer, to a final dilution 1/8 (w/v). For exposing the homogenates to ultraviolet irradiation 8 ml of the homogenate were placed in a 120 ml round bottom quartz flask. EDTA or other compounds were added to the flask in 1 ml of buffer and 1 ml of buffer was added to the controls. The flasks were rotated at 15 r.p.m. at a distance of 15 cm from the ultraviolet lamp. The ultraviolet source was a Hanovia Utility Model mercury arc lamp rated at an intensity of more than 250 microwatts per sq cm at a distance of 50.8 cm for wavelengths below 3130 Å. A current of air maintained the temperature at 27 ± 1°C. Samples were removed at intervals and peroxide formation determined by the TBA (thiobarbituric acid) reaction(6). Mitochondria from rat liver were prepared by differential centrifugation in isotonic sucrose(7). The fluffy layer was discarded and the mitochondria were washed twice with isotonic KCl and resuspended in isotonic KCl-Na-phosphate buffer, pH 7.4. Succinoxidase activity was measured in the mitochondria by the method of Schneider and Potter(8), using the equivalent of 36 mg of liver in each Warburg vessel. The average oxygen uptake of these preparations was 151 μl/30 min. The irradiation procedure was the same as that for the homogenates. For irradiation of methyl linolenate 0.05 ml of the ester was emulsified with 100 ml of M/10 phosphate buffer, pH 7.0, in a Waring blendor for 4 minutes. Ten ml of this emulsion were placed in the quartz flasks and various substances were added in 1 ml volumes. The final volume in each flask was brought to 12 ml with buffer. Samples were taken at intervals and peroxide formation was measured by the TBA test after extraction with chloroform.

Results. Lipide peroxide formation in liver homogenates is catalyzed by iron or ascorbic acid(9,10). A wide variety of chemical substances will inhibit this oxidation among which chelating agents appear to be the most effective on a molar basis. These substances also inhibit lipide peroxide formation in homogenates exposed to ultraviolet light (Table I). Irradiation of mitochondrial suspensions results in an increased peroxide formation and there is a corresponding decrease in succinoxidase activity(3). In studies on inhibition of peroxide formation in mitochondria, EDTA was chosen as the most suitable inhibitor because of its effect on the homogenate, and its lack of absorption of ultraviolet light. Peroxide formation in mitochondria ex-

* Aided by grant from U. S. Atomic Energy Com.

TABLE I. Effect of Various Compounds on Lipide Peroxide Formation in Irradiated Liver Homogenates.

Rat liver homogenates were diluted 1 to 8. Final concentrations of added compounds were 2.3 $\times 10^{-3}$ M glutathione, diethyldithiocarbamate, NaCN, 2×10^{-4} M (1) and 2×10^{-5} M (2) EDTA.

Time of irradiation (min.)	TBA optical density					
	Buffer	Glutathione	Diethyldithiocarbamate	NaCN	EDTA (1)	EDTA (2)
60	.175	.075	.035	.050	.020	.000
120	.530	.140	.085	.105	.000	.145

posed to ultraviolet light for 2 hours was inhibited. Inhibition also occurred in the controls which were rotated in the dark (Table II).

Succinoxidase activity is partially inhibited by 5 x 10^{-3} M EDTA. This concentration will inhibit the oxidation of lipids by ultraviolet light and there is little further loss of enzyme activity due to the action of ultraviolet light. However, at an EDTA concentration of 1.0 x 10^{-3} M there is no inhibition of succinoxidase and a significant protection of enzyme activity occurs when mitochondria are irradiated for different times. The degree of protection is correlated with the inhibition of peroxide formation (Fig. 1).

EDTA does not inhibit peroxide formation in pure methyl linolenate exposed to ultraviolet light. It prevents, however, the initial antioxidant effect of ascorbic acid and the catalytic effect of iron on this system (Table III). In tissue homogenates, ascorbic acid in low concentrations and iron catalyze peroxide formation and EDTA inhibits both (unpublished).

Discussion. The inability of EDTA to inhibit peroxide formation in pure methyl linolenate cannot be ascribed to its presence in the water phase and its consequent inability to come in intimate contact with the fatty acid ester, since ascorbic acid, a highly water soluble substance, acts as an antioxidant under the same conditions. It is therefore necessary to conclude that ultraviolet light produces peroxides in this system without the intervention of cations. In tissues, however, the situation is different. Although some peroxide is formed in the presence of EDTA, the rate

TABLE II. Inhibition by EDTA (1.2×10^{-3} M) of Peroxide Formation in Mitochondria.

Suspension was irradiated for 60 and 120 min. Controls were rotated in the dark.

Time of irradiation (min.)	TBA optical density			
	Not irradiated		Irradiated	
	Buffer	EDTA	Buffer	EDTA
0	.040	.030	.040	.030
60	.050	.035	.080	.040
120	.080	.030	.170	.090

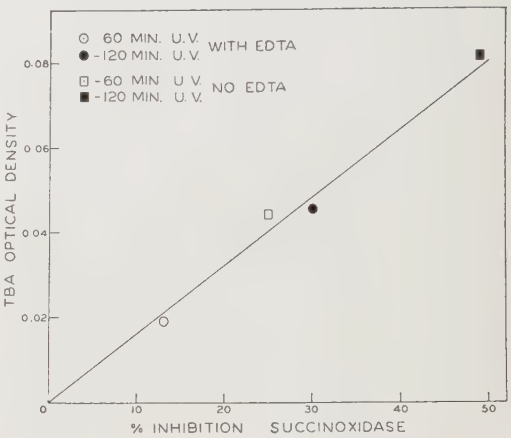

FIG. 1. Effect of EDTA (1.0×10^{-3} M) on peroxide formation and succinoxidase activity in rat liver mitochondria exposed to ultraviolet light.

TABLE III. Effect of Various Compounds on Peroxide Formation in Methyl Linolenate Exposed to Ultraviolet Light.

0.05 ml of methyl linolenate was emulsified in 100 ml of phosphate buffer M/10, pH 7.0, except in the case of iron where 100 ml of demineralized water was used. The final concentrations of added compounds were 1.2×10^{-3} M EDTA, 1.2×10^{-3} M ascorbic acid, and 1.0×10^{-4} M $FeCl_3 \cdot 6H_2O$.

Time of irrad. (min.)	TBA optical density					
	H_2O		Ascorbic acid		$FeCl_3$	
	H_2O	EDTA	H_2O	EDTA	H_2O	EDTA
0	.090	.070	.070	.070	.145	.095
10	.405	.380	.140	.380	.680	.455
20	.700	.700	.470	.670	.900	.620
30	.930	.945	.750	.945	1.155	.800
40	1.060	1.080	.920	1.160	1.300	1.000

of formation is greatly reduced by it. This suggests that cations may act catalytically when the unsaturated fats are associated with tissue components. The mechanism, however, is not clear. Most tissues contain antioxidants which may be chelating agents, from which a cation, possibly iron, could be released by the action of ultraviolet light to catalyze lipide peroxide formation. EDTA would chelate the cation and inhibit its catalytic activity. There is no explanation at present for the effect of EDTA on the antioxidant action of ascorbic acid on pure methyl linolenate.

Since the succinoxidase inhibition both in the presence and absence of EDTA is directly proportional to the lipide peroxide concentration, it appears that lipide peroxides mediate to a great extent the effect of ultraviolet light on this system.

Summary. 1. EDTA inhibits lipide peroxide formation in rat liver homogenates and in rat liver mitochondria exposed to ultraviolet irradiation. 2. Ultraviolet light inhibits succinoxidase in mitochondria and EDTA protects it from inactivation. The protection is proportional to the inhibition of lipide peroxide formation. 3. EDTA does not inhibit lipide peroxide formation in pure methyl linolenate emulsions irradiated with ultraviolet light but does inhibit both the antioxidant effect of ascorbic acid and the iron catalysis of peroxide formation in these emulsions.

1. Fisher, W. D., and Wilbur, K. M., *J. Elisha Mitchell Sci. Soc.,* 1954, v70, 124.

2. Stillwell, E. F., M. A. Thesis, Duke Univ., Durham, N. C., 1953.

3. Ottolenghi, A., Bernheim, F., and Wilbur, K. M., *Arch. Biochem. and Biophys.,* 1955, v56, 157.

4. Bernheim, F., Wilbur, K. M., and Kenaston, C. B., *ibid.,* 1952, v38, 177.

5. Wilbur, K. M., Kenaston, C. B., Wolfson, N., Ottolenghi, A., and Gaulden, M. E., *Anat. Rec.,* 1954, v120, 708.

6. Wilbur, K. M., Bernheim, F., and Shapiro, O. W., *Arch. Biochem.,* 1949, v24, 305.

7. Umbreit, W. W., Burris, R. H., and Stauffer, J. F., *Manometric Techniques and Tissue Metabolism,* Burgess Publishing Co., Minneapolis, 2nd Ed., 1951, p153.

8. Schneider, W. C., and Potter, V. R., *J. Biol. Chem.,* 1943, v149, 217.

9. Kuck, S. D., *Arch. Biochem.,* 1950, v24, 305.

10. Bernheim, F., Wilbur, K. M., and Fitzgerald, D. B., *J. Gen. Physiol.,* 1947, v31, 195.

Received August 12, 1957. P.S.E.B.M., 1957, v96.

Effect of Dietary Lipids on the Lipids in Rats' Milk.* (23511)

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It is well known (for reviews see 1,2,3) that the amount and type of fat in the diet of ruminants can affect the amount and particularly the composition of the milk fat. With monogastric animals these relationships have received less attention. Gogitidse(4) concluded that the iodine number of the milk fat increased after feeding of hemp seed oil or linseed oil to women. Mueller and Cox(5) reported that the iodine number of the milk fat of rats followed that of the fat fed, and that "this relation can be expressed as a straight line function." Loosli *et al.*(6) reported that feeding of corn oil or ethyl linoleate to lactating rats significantly increased the iodine number of milk fat recovered from the stomachs of the young immediately after

* Paper No. 3806, Scientific Journal Series, Minn. Agric. Exp. Station. Financial support for part of this work from the General Research Fund of the Graduate School, University of Minnesota, and from the Williams-Waterman Fund of Research Corp., N. Y., is gratefully acknowledged. The L-lysine hydrochloride was a gift from the E. I. du Pont de Nemours and Co., Wilmington, Delaware, and the B vitamins were generously furnished by Merck and Co., Rahway, New Jersey and the Lederle Laboratories, Pearl River, N. Y. Thanks are due to Mr. R. F. Derr for technical assistance.

TABLE I. Unsaturated Fatty Acid Content of Rations and Their Lipid Components.

Ration or lipid	Fatty acids,* g/100 g of diet or lipid		
	Dienoic	Trienoic	Tetraenoic
Ration AA_{20}	.283	none	none
" AA_{21}	1.49	.099	.003
" OC_4	3.60	.217	.015
Corn oil	58.8	1.00	.0
Wheat germ oil	48.2	4.42	.15
Fat from rolled oats	35.7	2.00	.21
Methyl linoleate-urea complex†	22.6	none	none

* Calculated as linoleic, linolenic and arachidonic acids respectively.
† Dried *in vacuo* at 50°C.

suckling.

Experiments in this laboratory(8) afforded an opportunity to determine the polyenoic acids in milk of rats which consumed diets containing either methyl linoleate or 3% of vegetable oil as the only source of fatty acids.

Methods. The milk was collected by the method of Luckey *et al.*(9) from rats towards the end of their second reproduction-lactation cycle. Milk donors and their dams had been fed exclusively ration AA_{20} or AA_{21}, previously described in detail(8), since weaning and throughout pregnancy and lactation. Ration AA_{20} was assembled from compounds of known chemical structure and contained sucrose, 16 amino acids, 10 salts, 14 vitamins, choline chloride, propylene glycol and 1.25% of methyl linoleate urea complex. Ration AA_{21} contained the same ingredients as ration AA_{20} except that 3% of a 2:1 mixture of wheat germ oil and corn oil was substituted for the methyl linoleate complex, alpha-tocopherol and part of the sucrose. The polyenoic acid content of rations and their lipid components are shown in Table I. Two specimens of milk fat were also available which had been collected from rats fed ration OC_4(10) which contained 84% rolled oats, 6.5% casein, 2% wheat germ oil, 1% corn oil, salts and vitamins. The milk was stored under nitrogen at −15°C until analyses were made. In a few instances fat of freshly collected milk was separated by centrifugation, washed with water and then stored as indicated. The milk was extracted 3 times under reflux and a slow stream of nitrogen with about 5 times its volume of a mixture of ethanol-diethyl ether (3:1) for 30 minutes at 70°C. Cooled extracts were filtered through Whatman No. 40 filter paper and the latter washed with more hot solvent. Ether was removed from combined extracts and washings at about 35°C with a stream of purified nitrogen, the alcoholic residue was diluted to a convenient volume and aliquots were used for determination of dry weight and for alkali isomerization. As suggested by Holman(11) the ethanolic solution of lipid was used directly for alkali isomerization which was carried out according to procedures of Herb and Riemenschneider(12). In accord with others(13), who have analyzed milk fat, preliminary experiments showed that saponification prior to alkali isomerization did not affect the results obtained with our specimens and it was not used. Measurements of absorbance at 233, 268, 315 and 346 mμ were made before and after alkali isomerization. Concentration of polyenoic acids was calculated by formula C of Herb and Riemenschneider(12) in terms of linoleic, linolenic and arachidonic acids although the chain lengths and the positions of double bonds of unsaturated fatty acids in rats' milk are admittedly not known. Absorbance before alkali isomerization, presumably due to the presence of conjugated polyenoic compounds, contributed in milk from rats fed ration AA_{20} 12.7 and 5.5% of the values calculated for unconjugated dienoic and trienoic acids respectively; with milk fat from rats fed ration AA_{21} the corresponding values were 3.8% and 3.6% respectively. The lipid components of the rations were also analyzed by alkali isomerization.

Results. Table II presents a summary of analyses for polyenoic acids in milk fat. Rats which were maintained on a diet devoid of trienoic and tetraenoic acids were able to secrete fat which, on the basis of spectrophotometric evidence, contained significant quantities of unconjugated trienoic and tetraenoic acids. When polyenoic acid content of the ration was increased (Table I) by addition of natural oils, total fat content of the milk was decreased by 28% but the milk fat had a greatly increased concentration of dienoic acid and a less marked but significant increase in trienoic

TABLE II. Effect of Diet on Unsaturated Fats in Rats' Milk.

Source of fat	No. or type of specimen	Fatty acids as % of total fat		
		Dienoic	Trienoic	Tetraenoic
Milk of rats fed ration AA_{20} (fat content of milk 12.2 ± .92%*)	22	1.86 ± .09*	.71 ± .01	.35 ± .03
Milk of rats fed ration AA_{21} (fat content of milk 8.8 ± .98%*)	16	6.66 ± .38*	1.02 ± .06	.84 ± .05
Milk of rats fed ration OC_4	Pooled, purified specimen†	17.6	1.37	1.08
Idem	Pooled, centrifuged fat‡	36.8	2.63	2.59

* Stand. error of mean.

† Specimen prepared by R. L. Glass from pooled rats' milk, deproteinized with equal vol of 95% ethanol, extraction of alcohol soluble fraction with 5 volumes of a mixture of diethyl ether and petroleum ether (1:1, v/v), drying over Na_2SO_4, removal of solvent and storage under nitrogen at −15°C.

‡ Specimen prepared by R. L. Glass from pooled rats' milk by centrifugation; used without further purification or extraction.

and tetraenoic acids. A 5.2-fold increase in dienoic acid content of the diet brought about a 3.6-fold increase in dienoic acid of milk fat.

Two specimens of pooled milk fat, from rats fed a ration of natural products which supplied 3.6% of dienoic acid, contained 17.6% and 36.8% of dienoic acid. In another specimen, collected from a single rat fed ration OC_4, extracted and analyzed as described above, concentrations of dienoic, trienoic and tetraenoic acids were 21.1%, 1.41% and 1.03% respectively. In comparison with fat from cows' milk for which a maximum value of 2.7% of dienoic acid has been reported(14) the content of the polyenoic acids in fat of rats' milk is unusually high. In the unpurified, pooled specimen of milk fat, from rats fed ration OC_4, which contained 36.8% of dienoic acid, the absorbance at 233 millimicrons before alkali isomerization was only 2.7% of that determined afterwards; hence, most of the dienoic acid appeared to be of the unconjugated type and of dietary origin.

The concentration of polyenoic acids in dietary fats reported in Table I falls into the range of values reported by others(15,16,17).

The chemical nature and origin of unsaturated fatty acids found in milk fat of rats fed ration AA_{20} cannot be ascertained with certainty from available evidence. The analytical methods used give no clue to maximum chain length of the polyenes. The dienoic acid is no doubt linoleic acid of dietary origin since the rat cannot synthesize this compound at a rapid rate. Holman(18) and Deuel and Reiser(19) have reviewed evidence for conversion of linoleic acid to arachidonic acid which, according to recent work(20) can apparently occur in the liver of the rat. We are not aware of evidence for conversion of linoleic acid to linolenic acid. However, Mead and Slaton(21) isolated recently from pooled tissues of 25 male rats, maintained on a fat-free diet, 118 mg of hexabromide of a compound assigned the structure of 5,8,11-eicosatrienoic acid. They suggest that this was formed by reduction of arachidonic acid and not as an intermediate in conversion of linoleic acid to arachidonic acid. Hence it might arise indirectly from linoleic acid. Dam *et al.* (22) have recently reported that total trienoic acid increased in tissues of rats during 18 weeks maintenance on a nearly fat-free diet and attributed this to conversion of linoleic to trienoic acid. If it is assumed that a litter of rats, while solely dependent on mother's milk, makes a weight increment of 200 g of fresh weight or 50 g dry weight(23) with a 35% efficiency(24) of conversion of milk solids into body solids, and that the fat content of rats' milk(25) is 40% of the milk solids, the total output of trienoic acid, during this period, of a rat fed ration AA_{20} would be about 0.4 g. This is about twice the amount of tetraenoic acid produced from linoleic acid (20,26) and greatly exceeds the quantity of the C_{20}-trienoic acid found in tissues of rats fed a fat-free diet(21). There is no assurance

that polyenoic acids found in milk fat from rats fed rations containing natural oils were the same as those observed when the methyl linoleate-urea complex was the only source of dietary fatty acid.

Microbiological synthesis of trienoic acid in the intestinal tract cannot be excluded as a possible source of polyenoic acid in milk fat although coprophagy has hitherto not been reported as a complicating factor in development of dietary deficiency of polyenoic acids. It appears more likely that trienoic acid was formed in the mammary gland or elsewhere, under stimulus of lactation, in amounts far exceeding those which non-lactating young rats can synthesize, if at all, on a fat-free diet.

These studies demonstrate clearly that concentration of dienoic and, to a smaller extent, of trienoic and tetraenoic acids in milk of a monogastric animal can be markedly elevated by an increased consumption of such compounds. Whether such an increase, over amounts needed to meet requirements for linoleic acid, is of nutritional benefit to the young organism is not known but this question merits attention with respect to possible long-range dietary effects of unsaturated fatty acids(27,28).

Summary. Spectrophotometric determinations of polyenoic acids showed that the milk of rats whose diet contained methyl linoleate as the only source of fatty acid contained significant quantities of unconjugated dienoic, trienoic and tetraenoic acids. Feeding of vegetable oils caused a great increase in concentration of unconjugated dienoic acid and, to a smaller extent, an increase of trienoic and tetraenoic acids in milk fat.

1. Deuel, H. J., 1955, *The Lipids,* Vol. II, p798, Interscience Publishers, N. Y.

2. Buschmann, A., *Tierernährung,* 1930, vI, 129.

3. Smith, F. H., Wells, C. A., and Ewing, P. V., *Georgia Agric. Exp. Sta. Bull.,* 1916, v122, 95.

4. Gogitidse, S., *Z. Biol.,* 1905, v46, 403.

5. Mueller, A. J., and Cox, W. M., Jr., *J. Biol. Chem.,* 1938, v123, LXXXVIII.

6. Loosli, J. K., Lingenfelter, J. F., Thomas, J. W., and Maynard, L. A., *J. Nutrition,* 1944, v42, 81.

8. Schultze, M. O., *J. Nutrition,* 1957, v61, 585.

9. Luckey, T. D., Mende, T. J., and Pleasants, T., *ibid.,* 1954, v54, 345.

10. Hallanger, L. E., and Schultze, M. O., *ibid.,* 1956, v60, 25.

11. Holman, R. T., Hormel Foundation, University of Minnesota, Annual Report, 1955-56, p43.

12. Herb, S. F., and Riemenschneider, R. W., *Anal. Chem.,* 1953, v25, 953; *J. Am. Oil Chem. Soc.,* 1952, v29, 456.

13. McDowell, A. K., *J. Dairy Research,* 1954, v20, 101.

14. Jack, E. L., and Smith, M. L., *J. Dairy Sci.,* 1956, v39, 1.

15. Firestone, D., *J. Am. Oil Chem. Soc.,* 1955, v38, 657.

16. Willard, C., Englert, R. D., and Richards, L. M., *ibid.,* 1954, v31, 131.

17. Radlone, S. B., *Oil and Soap,* 1945, v22, 183.

18. Holman, R. T., in Sebrell W. H., Jr., and Harris, R. S., *The Vitamins,* 1954, vII, 280, Academic Press, Inc., N. Y.

19. Deuel, H. J., Jr., and Reiser, R., *Vitamins and Hormones,* 1955, v13, 29, Academic Press, Inc., N. Y.

20. Steinberg, G., Slaton, W. H., Jr., Howton, D. R., and Mead, J. F., *J. Biol. Chem.,* 1956, v220, 257.

21. Mead, J. F., and Slaton, W. H., Jr., *ibid.,* 1956, v219, 705.

22. Dam, H., Engel, P. F., and Nielsen, G. K., *Act. Physiol. Scand.,* 1956, v37, 143.

23. Annegers, J., PROC. SOC. EXP. BIOL. AND MED., 1954, v87, 454.

24. Brodie, S., *Bioenergetics and Growth,* p57, Reinhold Publishing Corp., N. Y., 1945.

25. Glass, R. L., Ph.D. Thesis, University of Minn., 1956.

26. Widmer, C., Jr., and Holman, R. T., *Arch. Biochem.,* 1950, v25, 1.

27. Carroll, K. K., PROC. SOC. EXP. BIOL. AND MED., 1957, v94, 202.

28. Van Italie, T. B., *J. Am. Diet. Assn.,* 1957, v33, 352.

Received August 15, 1957. P.S.E.B.M., 1957, v96.

A Fluorescent Test for Treponemal Antibodies. (23512)

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The value of fluorescent antibody procedures has been adequately demonstrated in a wide variety of studies(1,2,3). Until recently, these technics have not been applied in the field of venereal disease(4). It is the purpose of this report to describe and discuss the use of fluorescein labeled antiglobulin as an indicator of treponemal antibodies.

Materials and methods. Treponema pallidum, Nichols strain (T.p.) was used as an antigen and was obtained from rabbit testicular tissue by extraction as recommended for the *Treponema pallidum* Immobilization (TPI) test(5). Human serums were made available from laboratory stocks. Syphilitic rabbit serums were produced by intradermal inoculation of minimal infective doses of T.p. and bleeding from ear veins at weekly intervals. All serums were heated at 56°C for 30 minutes before being tested. Dilutions of serums and globulins were prepared in phosphate buffered saline pH 7.2 (BS). Production of goat antihuman serum (GAH) and goat antirabbit serum (GAR) followed the general procedure as outlined by Proom(6). Nanny goats of about 12 months of age were injected according to the following schedule: 1st intramuscular injections consisted of 5 ml potassium alum serum precipitate in each leg. The 2nd injection 2 weeks later consisted of 10 ml of precipitate into one leg. The 3rd injection, four weeks after the 1st, was 10 ml into one leg. Following a one-month rest, 10 ml whole serum was injected subcutaneously in the region of the back. One week following this injection, the animals were bled from the jugular vein. Globulins were prepared by repeated precipitation with half saturated ammonium sulfate and were dialyzed against BS. The protein content of this preparation was adjusted to a concentration of 1.5 g/% for subsequent fluorescein conjugation by the technics of Coons and Kaplan(7).* Finished conjugates were twice precipitated with ammonium sulfate and finally dialyzed against BS. Fluorescent microscopy was accomplished by use of Leitz and Reichert ultraviolet light assemblies and the appropriate filters. Microscopic observations were made using darkfield condensers and 4 mm. dry objective.

Test Procedure, Method A. T.p. suspensions demonstrating 2-10 organisms/H.P. microscopic field were used for slide preparations. Freshly extracted T.p. suspensions, or suspensions held in a refrigerator at 4°C without added preservative, were used with equal results. Smears were prepared on 3 x 1 inch microscope slides. A diamond point stylus was used to cut circles which would identify smear areas for later observations. A drop of T.p. suspension (antigen) consisting of approximately 0.01 ml was formed into a circular smear of about 10 mm diameter. Slides were air dried and gently fixed with heat. Approximately 0.03 ml of 1-5 dilution of serum (heated at 56°C for 30 minutes) was placed on each smear and spread in a circular fashion. Loss of moisture by evaporation was prevented by covering the slides with large Petri dish tops containing moistened filter paper. Smears were allowed to be in contact with serums for 30 minutes, at which time they were carefully rinsed with BS and were then allowed to stand in BS for 10 minutes. Excess moisture was drained from slides and smears were gently blotted. Fluorescein labeled GAH diluted 1-3, or GAR diluted 1-5 was placed on the smears in the same amount and manner described for serums. Slides were protected with a Petri plate as before for 30 minutes. At the end of this period, slides were washed and soaked for 10 minutes as previously described. Following this treatment, slides were carefully wiped free of moisture, but only the excess moisture was removed by shaking. A very small drop of mounting mixture consisting of one part

* The authors wish to express their appreciation to Dr. Morris Goldman, Laboratory Branch, CDC, for preparation of fluorescein conjugates used. Fluorescein amine was prepared by Mr. I. Green.

TABLE I. Rabbit Experiment Illustrating Comparative Test Results.

Rabbit No.	No. TP inoculated	Days after inoculation	Serologic tests: Fluorescent antibody methods A	B	TPI	TPCF	VDRL slide
631	10^4	0	—	—	—	—	—
		14	—	—	—	—	—
		35	—	—	—	—	—
		42	—	—	—	—	—
		49	—	WR	—	R	—
		64	—	R	—	R	—
		112	—	R	—	R	—
630	10^3	0	—	—	—	—	—
		14	—	—	—	—	—
		35	—	—	—	WR	WR
		42	—	WR	—	WR	WR
		49	—	R	—	R	R
		64	WR	R	—	R	R
		112	R	R	WR	R	R
M12	10^5	0	—	—	—	—	—
		7	—	—	—	—	—
		14	—	WR	—	—	—
		21	—	R	—	R	R
		49	WR	R	R	R	R
		64	R	R	R	R	R

R = reactive; WR = weakly reactive; — = (NR) nonreactive.

buffered saline pH 7.2 and nine parts C.P. glycerine was placed on each smear, and the slide preparation was completed by placing a cover glass on the mounting medium. Controls consisted of mounted untreated T.p. smears, T.p. stained only with labeled GAH or GAR, and with known normal and syphilitic serums. Slides were examined immediately after preparation, but were stored at 4°C for several weeks for later reference. *Test Procedure, Method B.* This method was in every respect similar to Method A except that slides were placed on a mechanical rotator at 160 rpm during the 30 minute periods that the treponemes were exposed to the serum dilutions and the fluorescein labeled globulin preparations.

Reading of test controls and serums. All smear preparations were examined by regular darkfield illumination and by means of filtered light from a high pressure mercury vapor bulb. Properly prepared untreated smears demonstrated well defined T.p. when visible illumination was used. The same field gave negative results when viewed with ultraviolet light (UV). Smears treated with labeled GAH or GAR only, or those treated with normal serums followed by treatment with fluorescein labeled preparations, demonstrated characteristics similar to those of untreated smears. Smears which had been treated with human or rabbit serums containing treponemal antibodies revealed fluorescent T.p. following the application of GAH or GAR. Positive reactions were observed to vary from strong to weak fluorescence. Results obtained by the fluorescent treponemal antibody test (FTAT) from syphilitic rabbits are illustrated in Table I. Findings obtained by means of other treponemal test procedures are also recorded for comparative purposes. The VDRL Slide test(8) results are listed as an example of reactions obtainable by a nontreponemal test. As will be observed, the reactions recorded for FTAT Method A are in only slight agreement with either FTAT Method B or the other test procedures. It will also be noted that more reactions were obtained by Method B than by Method A. Since rotation of slides is the only difference between these methods, it appears that this may account for the marked intensification of fluorescent tagging by this procedure.

Discussion. It is evident from the experimental results presented that the use of intact T.p. as an antigen and fluorescein labeled

TABLE II. Examples of Testing Discrepancies with Human Serums.

Serum No.	Serologic tests: FAST* method "B"	TPI	TPCF	VDRL slide
1456	R	R	R	R
1457	R	R	R	R
1459	R	R	R	WR
1468	—	—	WR	WR
1378	WR	R	WR	—
1477	R	—	R	—
1498	R	—	AC†	—
1501	R	R	AC	—
1503	R	—	AC	R
1512	R	R	WR	R
2013‡	R	incl.§	R	R
2028‡	—	—	AC	WR
2060‡	R	R	R	R
2062‡	R	R	R	R

R = reactive; WR = weakly reactive; — = (NR) nonreactive.

* Fluorescent Antibody Slide Test Method B.

† Anticomplementary.

‡ Serums demonstrated inconclusive (incl.) results in the TPI test before penicillinase treatment.

§ Control shows TP toxicity even after penicillinase treatment.

antiglobulin as an indicator of treponemal antibody coupling is a wholly feasible procedure. In the rabbit inoculation experiment, Table I, Method B appears to follow the course of immunologic response to T.p. infection in an acceptable manner. Method B and the TPCF test are apparently similar in respect to sensitivity, whereas Method A and the TPI procedure gave comparable results. The reactivity of Method B is further illustrated in Table II in which the results obtained by the 3 T.p. test procedures on human serums are compared. The serums in this series were selected to illustrate various combinations of reactivity found in a much larger group of serums tested. Reactivity in only one of the three T.p. tests is illustrated by serum No. 1468. Serums No. 1498 and 1503 were found to be reactive only by the FTAT Method B. Serums No. 2013, 2028, 2060 and 2062 were selected to illustrate that the FTAT Method B may demonstrate reactive results when the other T.p. tests have yielded inconclusive findings. It should be noted that specimens found to be anticomplementary in the TPCF test and inconclusive in the TPI test, due to penicillin or unknown toxic factors, may yield positive findings in the fluorescent antibody procedure.

In consideration of the practical application of the fluorescent treponemal antibody test Method B to syphilis serology, it must be emphasized that the specificity of the reaction has not yet been determined in terms of human disease. Such an evaluation is currently under consideration. It is evident, however, that syphilis infection in the rabbit gives rise to an antibody or antibodies that may be detected by fluorescent antibody testing. As previously mentioned, the fluorescent antibody testing procedure herein described compares favorably with the TPCF test in terms of sensitivity, and appears to be considerably more sensitive than the TPI procedure. If the reaction obtained by the fluorescent antibody testing method should prove to be adequately specific, several other important factors would recommend the method as a possible laboratory procedure. Among these may be included the following: the procedure is not as expensive as some T.p. tests, and materials can be made available from commercial sources; the actual reading of an individual test consumes approximately one minute and the entire test can be completed in about one hour; slides may be examined at a convenient time and filed for future reference under refrigeration (4°C) for several weeks. As previously stated, the validity of results obtained with the fluorescent antibody test in terms of human infection must await more complete documentation. Therefore, it is too early to speculate on the immunological significance of the antibodies detected by this means.

Summary. 1. A technic for the use of fluorescein labeled antiglobulin as an indicator of treponemal antibody is described. 2. The effect of rotation as an intensifier of fluorescent antibody reactions is determined. 3. Results obtained with fluorescent antibody tests on syphilitic rabbits are compared with results of other treponemal and nontreponemal tests. 4. Serological discrepancies obtained with fluorescent antibody and several other tests on human serums are presented.

Addendum. Since this article was offered for pub-

lication it has been found that extraction of treponeme smears with acetone for 15 minutes prior to use of serum reduces background fluorescence so is a preferred method.

1. Coons, A. H., Creech, H. J., Jones, R. N., and Berliner, E., *J. Immunol.*, 1942, v45, 159.

2. Goldman, Morris, *Am. J. Hyg.*, 1953, v58, 319.

3. Moody, Max D., Goldman, Morris, and Thomason, Berenice, *J. Bact.*, 1956, v72, 357.

4. Harris, Ad, Deacon, W. E., and Smith, C. A., *Bull. Conf. of State & Prov. P. H. Lab. Directors*, 1957, v15, 8.

5. Harris, Ad, Bossak, H. N., Olansky, S., *Pub. Health Lab.*, 1955, v13, 63.

6. Proom, H., *J. Path. Bact.*, 1943, v55, 419.

7. Coons, A. H., and Kaplan, M. H., *J. Exp. Med.*, 1950, v91, 1.

8. Serologic Tests for Syphilis, 1955 Manual, PHS Publ., No. 411, Washington, D. C.

Received August 16, 1957. P.S.E.B.M., 1957, v96.

Paroxysmal Nocturnal Hemoglobinuria. Evidence of Defect of Red Cell Stroma Manifested by Abnormalities of Lipids. (23513)

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Paroxysmal nocturnal hemoglobinuria (PNH) may be a manifestation or symptom of a chronic hemolytic anemia characterized by an abnormality of the red blood cell which renders it susceptible to hemolysis by the action of the "properdin system." The components of this system are normal constituents of the plasma namely properdin, Mg^{++} and cofactors resembling complement(1). The system apparently is not active at all against normal human red cells. A great amount of work has been done to study and define the plasma factors of the PNH hemolytic system, but the erythrocyte which carries the pathognomonic defect has received little attention except as an indicator of the reactions of the plasma factors. It has been accepted that the PNH erythrocyte is faultily constituted, but the nature of fault is unknown(2). The hemoglobin has a normal solubility and electrophoretic mobility. It has been suggested that the defect resides in the red cell stroma or in the metabolic machinery that maintains the stroma throughout the life span of the cell. The present study was undertaken to learn whether or not the stromal lipids of the PNH erythrocyte are different from those of normal red cells.

Materials and methods. The blood for these determinations was obtained from 2 patients with classical history and signs of PNH. Patient A has been known to have the disease since 1939. During the past 10 years he has gradually improved until at present he shows no evidence of hemolytic disease or anemia. His red cells, though giving the characteristic reactions *in vitro*, survive normally when transfused into the patient or a normal recipient. Patient B has a serious hemolytic disease, with anemia and hemoglobinuria, and 15 per cent of his red cells are reticulocytes. Control studies were done on the red cells of 14 normal adults. The lipid analyses were performed on the washed red cells. The details of the procedure are the subject of a separate report. In brief, the lipids were extracted by boiling ethanol-ether and the analysis was carried out under nitrogen. Total fatty acids were determined by weighing. Saturated fatty acids were precipitated as the lead salts and weighed (3,4). Polyethenoid fatty acids were measured by the alkali-isomerization technic of Herb and Riemenschneider(5,6). Oleic acid was computed to be the difference between the total fatty acids and the sum of the saturated and polyethenoid acids. Iodine number was determined by the Yasuda procedure(7). Cholesterol was determined by a standard method(8,9). Recovery experiments and parallel and segmental analyses on red blood cells from the same patient permit a high degree of confidence in the methods and results. The mean corpuscular area was determined by the photomicrography of fresh red cells in rouleaux by an original method which gives a high degree of accuracy. Mean corpuscular volume

TABLE I. Red Cell Lipids from 2 Patients with PNH Related to Cell Surface Area and Compared with Values from 14 Normal Patients.

	Normal ranges	Normal avg	PNH Case A	PNH Case B
Cell vol (μ^3)	74–92	82	105	80
Surface area (μ^2)	128–144	135	160	150
Fatty acids				
Iodine No.	99–126	112	175	121
Total fatty acids				
mg/ml cells	1.2–2.2	1.7	1.2	1.7
mg/$10^{-14}/\mu^2$	74–135	105	79	91
Unsaturated F.A.				
Linoleic (% total)	4.4–7.3	5.7	8.6	5.2
(mg $10^{-14}/\mu^2$)	4–9	6	7	5
Linolenic (% total)	0	0	0	0
(mg $10^{-14}/\mu^2$)	0	0	0	0
Arachidonic (% total)	14.8–19.4	17.1	22.9	22.2
(mg $10^{-14}/\mu^2$)	11–24	18	18	20
Pentaenoic (% total)	2.7–4.4	3.6	5.8	4.6
(mg $10^{-14}/\mu^2$)	2–5	4	4	4
Hexaenoic (% total)	2.1–6.1	3.8	5.2	5.4
(mg $10^{-14}/\mu^2$)	2–8	4	4	5
Total polyethenoid (% total)	24.0–37.2	30.2	42.5	37.4
(mg $10^{-14}/\mu^2$)	19–46	32	33	34
Oleic (% total)	41.8–57.5	48.2	39.9	29.8
(mg $10^{-14}/\mu^2$)	34–60	50	32	27
Saturated F.A. (% total)	13.5–28.1	22.5	17.6	32.8
(mg $10^{-14}/\mu^2$)	14–31	23	14	30
Free cholesterol (mg %)	117–158	132	143	152
(mg $10^{-14}/\mu^2$)	79–99	80	92	81

was computed from these same measurements. The mean corpuscular lipid was related to the surface area because most if not all of the lipids are found at the surface of the red cell (10).

Results. (Table I). Abnormalities were found in the red cells of both patients: an increase in the concentration of arachidonic, pentaenoic and total polyethenoid fatty acids and a decrease in the concentration of oleic and total fatty acids. There were differences between cases A and B. In A the linoleic acid and the iodine number were high, in B they were normal. In A the saturated fatty acids were normal, in B they were high. In both cases the unesterified cholesterol was normal as was the concentration of the hexaenoic acids.

Both patients had macrocytosis and the surface area of the red cells was well beyond the normal range. When the abnormalities of the fatty acids are related to the surface area a different picture is obtained. The amount of fatty acid per μ^2 of surface is abnormally low because of the great surface area. Those highly unsaturated fatty acids which were found to be increased (arachidonic and pentaenoic) have normal values when computed in terms of mg per μ^2 of surface. The same is true for the saturated fatty acids in case B, but they are low in case A. Oleic acid which in both cases was low in terms of total amount is lower still when expressed as mg per μ^2 of surface.

Discussion. The fatty acids of normal red cells are concentrated at the surface in an amount sufficient to permit a bimolecular layer(10). The red cell fatty acids are structural in function and are probably present as components of the stromal lipoprotein. In PNH red cells the concentration of the total fatty acids is somewhat low but it lies within the normal range. However, the composition of the total is abnormal. The ratio of arachidonic and pentaenoic acids is high, and the total as well as the ratio of oleic acid is low. This means that the lipoproteins of the surface of the PNH red cell have different components from those of normal red cells. Whether or not this difference is related to the "PNH lesion" is not known.

The red cells of the 2 PNH patients differed from one another in at least 2 ways. The cells of patient B were more susceptible to the PNH hemolytic system, both *in vivo* and *in vitro*. Therefore the PNH lesion in case B may be said to have been more severe. The second difference is a consequence of the first. The red cells of patient B were much younger than those of patient A. Patient B had a severe hemolytic disease and his cells were rapidly turned over. They are estimated to have had an average age of less than 10 days. Patient A had cells that survived normally and their average age is estimated to have been 60 days. Lipid analyses of red cells in

other hemolytic anemias and in anemia due to hemorrhage have shown that young red cells are characteristically different from mature cells. The concentration of total fatty acids related to the surface of normal cells is the same in both, but the younger cells have a relatively high amount of saturated fatty acids. In comparing the 2 cases of PNH the outstanding difference is in the amount of saturated fatty acid. It is at the top of the normal range in case B, and at the bottom in case A. The difference is one that might be due to the ages of the cells rather than the severity of the PNH lesions.

Summary. The extracted lipids from the red cells of 2 patients were analyzed for cholesterol, unsaturated and saturated fatty acids. The ratios of the various lipids were established and the values were related to the total lipids and also to the surface area of the red cells. Compared with normal red cells the PNH cells showed an increased concentration of arachidonic and pentaenoic acids and a decreased concentration of oleic acid. These results suggest that the lipid pattern of the lipoproteins in the stroma of PNH cells is abnormal.

1. Hinz, C. F., Jordan, W. S., and Pillemer, L., *J. Clin. Invest.,* 1956, v35, 453.
2. Crosby, W. H., *Blood,* 1953, v8, 769.
3. Sinclair, R. G., *J. Biol. Chem.,* 1935, v111, 261.
4. Gorbach, G., *Fette u Seifen,* 1944, v51, 94.
5. Herb, S. F., and Riemenschneider, R. W., *Anal. Chem.,* 1953, v25, 953.
6. Hammond, E. G., and Lundberg, W. O., *J. Am. Oil Chem. Soc.,* 1953, v30, 433.
7. Yasuda, M., *J. Biol. Chem.,* 1931, v94, 401.
8. Schoenheimer, R., and Sperry, W. M., *ibid.,* 1934, v106, 745.
9. Sperry, W. M., and Webb, M., *ibid.,* 1950, v187, 97.
10. Ponder, E., *Blood,* 1954, v9, 227.

Received August 26, 1957. P.S.E.B.M., 1957, v96.

Hypothermic Effect of Chlorpromazine, Histamine and Serotonin, and Acclimatization to Cold. (23514)

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Recent studies have shown serotonin and histamine to be some of the products of secretion of mast cells(1). We have reported definite changes in the appearance and abundance of these cells in animals exposed to low temperatures(2). Since chlorpromazine is believed to be a "serotonin liberator"(3), its effect was tested in animals which had been exposed to cold for varied periods of time. Similarly the effect of cold acclimatization on the body temperature response to serotonin and histamine was investigated.

Methods. Each group of animals was composed of 8 male rats weighing approximately 150 g. All injections were intraperitoneal in a 1% solution. In the first experiment 4 groups, after being exposed to cold (6°C) for 0, 1, 7 and 28 days respectively, were maintained at 21°C for 1 day and were then given an injection of 10 mg/kg chlorpromazine. In a second experiment 2 groups exposed to cold (6°C) for 0 and 28 days were injected simultaneously with 100 mg/kg of histamine phosphate and 2 mg/kg of serotonin. Immediately following these 2 injections, 4 doses of 2 mg/kg of serotonin were injected at 5 minute intervals. In a third experiment with 2 groups exposed to cold (6°C) for 0 and 24 days, 5 doses of 32.4 mg/rat of histamine phosphate, were injected at ½ hour intervals. The body temperature was measured with a thermocouple inserted at a depth of 40 mm.

Results. Fig. 1 shows that the intensity and duration of the hypothermic effect of chlorpromazine is greatly decreased by pre-exposing animals to cold for 7 and 28 days. No attempt was made to quantitate the chlorpromazine effect in terms of serotonin and histamine response. However, as shown in Fig. 1, the effect of acclimatization on the body tem-

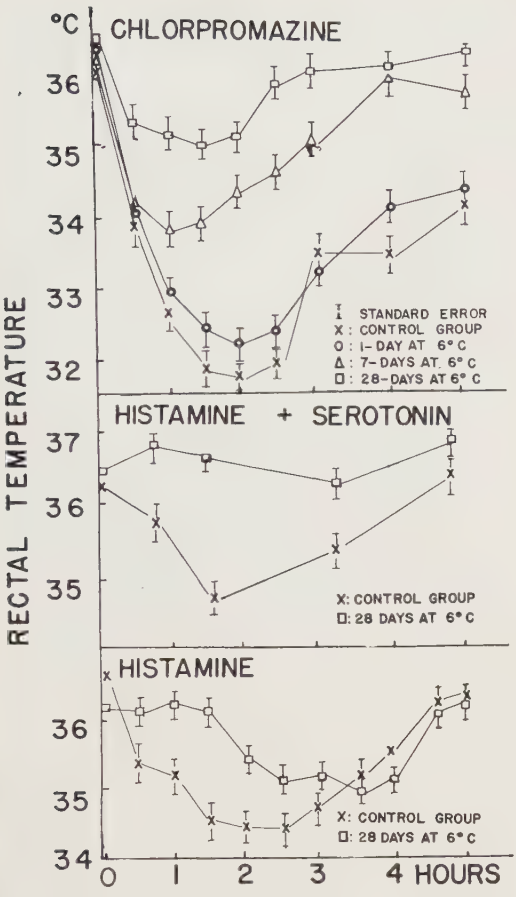

FIG. 1. Rectal temperature of rats previously exposed to cold, measured at 21°C following injections of chlorpromazine, histamine and serotonin.

perature response to histamine alone or combined with serotonin is qualitatively the same as the one observed with chlorpromazine. With the dose of chlorpromazine employed all control animals were unable to stand on their 4 legs, whereas the cold acclimatized animals (7 and 28 days) were able to walk.

Discussion. If the depressing effect of chlorpromazine on body temperature were due to a release of histamine and serotonin from mast cells, the alleviating effect of acclimatization would be caused by a decreased secretion of these substances, a diminished response of the organism to them, or by a combination of these 2 conditions. Since the effect of serotonin and histamine injections is also alleviated by acclimatization, it would seem then that the diminished response of the organism to these hypothermic substances is an important reason for the observed loss of hypothermic effect of chlorpromazine after cold acclimatization. Decreased endogenous production of serotonin and histamine is not excluded, but may not be needed to explain the effect of cold acclimatization on chlorpromazine. In conjunction with this it is interesting to note that in a recent study we have shown that mast cells, except in the extremities, increase in number in acclimatized animals(2). This result could indicate that a sufficient amount of serotonin and histamine is available in acclimatized animals to allow the hypothermic effect of chlorpromazine to take place.

Summary. The hypothermic effect of chlorpromazine, serotonin and histamine was investigated in rats at 21°C after acclimatization at 6°C for 1 to 28 days. The response to chlorpromazine was greatly reduced in animals acclimatized to cold for 7 and 28 days. Similarly, the fall in body temperature observed with serotonin and histamine was not as pronounced in acclimatized as in control animals.

1. Benditt, E. P., Wong, R. L., Arase, M., and Roeper, E., PROC. SOC. EXP. BIOL. AND MED., 1955, v90, 303.

2. LeBlanc, J., and Rosenberg, F., PROC. SOC. EXP. BIOL. AND MED., 1957, v96, 234.

3. Cook, L., and Weidley, E., *Annals N. Y. Acad. Sci.*, 1957, v66, 740.

Received August 26, 1957. P.S.E.B.M., 1957, v96.

Free Living Amoebae as Contaminants in Monkey Kidney Tissue Culture. (23515)

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During routine use of trypsinized monkey kidney tissue culture for viral research, "spontaneous" contamination of the tissue culture (TC) with a free living amoeba occurred twice in 1956 in our laboratory. Such a rare contamination, to the best of our knowledge, has never been reported. The isolation and the characteristics of the organism in TC are described in this paper.

Materials and methods. Tissue culture. Primary cultures of trypsinized monkey kidney cells were prepared by the method of Dulbecco and Vogt(1) as modified(2) by Youngner. Serial passage cultures of HeLa cells, Chang's liver cells and bovine kidney cells(3) were purchased from Microbiological Associates, Inc. Primary cultures of human amnion cells were kindly provided by Dr. Kenneth Takemoto(4) and a serial passage culture of human skin cells was obtained from Dr. Wilton R. Earle(5). Primary cultures of trypsinized chick embryo cells and rabbit kidney cells were prepared in a manner similar to the monkey kidney tissue culture. All these cell lines were cultured in roller tubes. The media used for these cell lines were either Eagle's basal medium with i-inositol(6,7) or Medium 199, except for monkey kidney cells, for which 0.5% lactalbumin hydrolysate in Hanks' balanced salt solution was used(8). Two per cent calf serum was added to all the media. For passage usually 0.2 ml of TC fluid containing the amoebic cysts or both cysts and trophozoites was transferred to each culture tube containing 1.8 ml of medium. Infected tissue cultures kept either stationary or in a rotating drum were incubated at 35°C. *Photography.* Photographs of unstained specimens were taken from the roller tubes. For stained specimens, the amoebae were propagated in TC on cover slips lying on the flat wall of Leighton tubes. After incubation the medium was poured off, 10% formalin was poured into the tube for fixation, and then the cover slip was removed for staining.

Results. Isolation of the Amoebae. Two strains of apparently the same amoeba were isolated from monkey kidney tissue cultures which showed cellular degeneration spontaneously on routine microscopic examination. Subcultures were made on monkey kidney tissue cultures and the same phenomenon was noted. Under low power (30X) microscopic examination, rounded bodies were observed, which were similar in appearance to cells manifesting changes induced by certain simian viruses (Fig. 1). When the specimen was examined under higher magnification (150X) these round bodies appeared not to be cells but cysts (Fig. 1), some of which were found to be intracellular. A hanging drop preparation was made on a slide from the TC fluid and examined microscopically with high magnification (450X). Moving amoebae were found and identified as free living amoebae belonging to the genus *Acanthameba.*†

Culture characteristics. The amoebae multiplied very well in cultures of HeLa cells, Chang's liver cells, human amnion cells, human skin cells, chick embryo cells, beef kidney cells, rabbit kidney cells and monkey kidney cells. Monkey kidney cells were least susceptible, while HeLa cells and chick embryo cells were most susceptible. After inoculation with the amoebae, the various cell lines did not undergo any specific degeneration that we could see. They became granular and gradually disintegrated and disappeared. When the cysts were inoculated into monkey kidney tissue culture, trophozoites were not seen until 5 to 8 days after inoculation. In cultures of HeLa cells or chick embryo cells, trophozoites were found 24 hours

* U. S. Department of Health, Education, and Welfare, Public Health Service.

† We are indebted to Dr. Leon Jacobs, Natl. Inst. of Allergy and Infectious Diseases, for these findings.

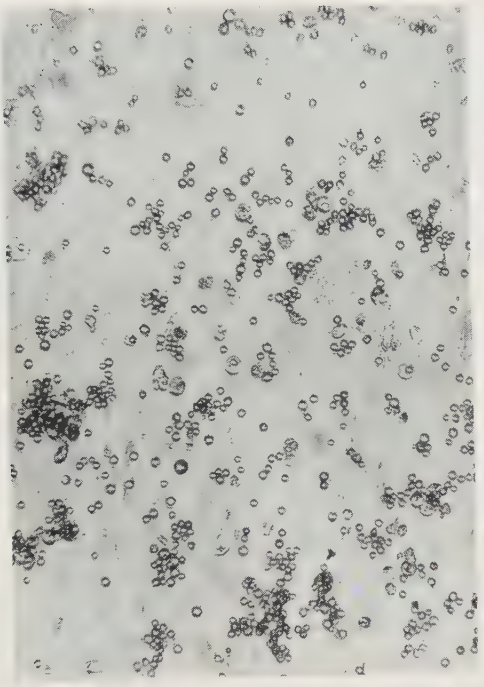

FIG. 1. Free living amoebae in monkey kidney tissue culture, early stage, showing cysts. × 90.

after inoculation, and the cells were destroyed in 24 to 48 hours. The sensitivity of the cells also depended on the age of the cells and the size of the inoculum. Older cells were more susceptible. Various culture media including Eagle's basal medium, Medium 199, and lactalbumin hydrolysate medium were used for cultivation and no difference was observed. The amoebae did not multiply in the absence of cells regardless of the media used. (When the TC fluid containing the amoebae was poured into boiled tap water to which rice powder was added and in which bacteria were present, the amoebae multiplied therein.)† The usual titer for cytotoxic changes in TC was 10^4 or 10^5 per 1.0 ml. By direct counts of the cysts in the TC fluids, there were usually 200,000 or more amoebae per 1.0 ml. One strain was transferred consecutively in monkey kidney tissue culture for 16 passages, and the other strain for 12 passages. Both strains were transferred in HeLa cells for more than 10 passages.

In some experiments 10-fold serial dilutions were made from the monkey kidney tissue culture fluid containing the amoebae, and the dilutions were inoculated into fresh monkey kidney tissue culture tubes, which were examined from day to day for presence of cysts and trophozoites. As shown in Table I, cysts appeared first, and they were gradually replaced by trophozoites. Toward the end of the observation period, as the tissue cells were all destroyed, all the trophozoites again became encysted.

In the monkey kidney tissue culture the cysts were found both outside and inside of the cells. In the cells they were found in the cytoplasm usually pushing the nucleus of the cell to the side (Fig. 2). From 1 to 28 cysts could be found in a single cell. In HeLa cells or chick embryo cells, intracellular amoebic

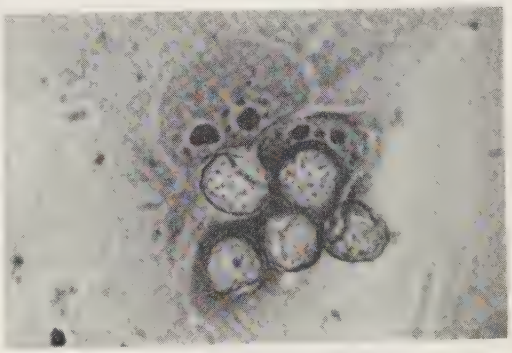

FIG. 2. Free living amoebae in monkey kidney culture, stained with azure eosin, showing wall of intracellular cysts. × 840.

TABLE I. Multiplication of a Free Living Amoeba in Monkey Kidney Tissue Culture.

Conc. of T.C. fluid inoc.	Observation of cysts and/or trophozoites on different days after inoculation													
	3		6		8		9		11		16		20	
	Cy	Tr	Cy	Tr	Cy	Tr	Cy	Tr	Cy	Tr	Cy	Tr	Cy	Tr
10^{-1}	+	—	++	—	++	±	+++	++	—	+++	+	+++	++++	—
10^{-2}	+	—	++	—	++	±	++	++	—	+++	+	+++	++++	—
10^{-3}	+	—	++	—	++	±	++	++	—	+++	+	+++	++++	—
10^{-4}	±	—	+	—	++	±	++	++	—	+++	+	+++	++++	—
10^{-5}	—	—	—	—	—	—	++	—	—	+++	+	+++	++++	—

+, ++, etc., indicate relative No. of amoebae found. — = amoebae not found. ± = questionably found.

Cy = amoebic cysts. Tr = trophozoites.

cysts were occasionally found. The cyst had a heavy double wall, the inner one rugose. The cytoplasm of the cyst was granular. No nucleus of the cyst could be seen when it was examined in TC tubes. The cyst varied in size, usually from 10 to 21μ in diameter, as measured from material taken from the monkey kidney tissue culture.

In one of the experiments the cysts were treated with 10% formalin and were then washed and inoculated into monkey kidney tissue culture tubes. These cysts could be found inside the cells. This observation suggested that the monkey kidney cells could phagocytize the cysts.

The trophozoites varied from 7 x 12 to 16 x 43 μ in diameter. They were sluggish in movement. A trophozoite produced many small pseudopodia. Many granules and food vacuoles were found in the cytoplasm with one or more contractile vacuoles. A contractile vacuole appeared when the amoeba was put into tap water and examined in hanging drop preparations microscopically. This was the chief reason why it was identified as a free living amoeba. Intracellular trophozoites were not observed.

Stability. The trophozoites were very sensitive to temperature. As soon as they were placed in the refrigerator at 4°C they became encysted. The cysts were very resistant to low temperatures. Culture fluids containing the cysts kept at −50°C for 8 months still yielded trophozoites when inoculated into TC tubes, although the titer was greatly reduced. The amoebae remained viable in the pH range 5.0-9.0 and were not affected by streptomycin and penicillin in TC.

Filtration experiment. Fluids of monkey kidney tissue culture and chick embryo tissue culture containing the cysts and trophozoites were filtered through an ultra-fine sintered glass filter or a S-3 Seitz filter. Ten-fold serial dilutions were made from the filtrate, and the dilutions, from 10^{-0} to 10^{-7}, were inoculated into monkey kidney tissue culture tubes and HeLa cell TC tubes. No cytopathogenicity or amoebae were found after seven or more days of incubation. The unfiltered fluid yielded positive cultures of amoebae at the concentration of 10^{-5}. These experiments are evidence that a virus was not present together with the amoebae.

Mixed infection of the amoebae and poliovirus in tissue culture. Monkey kidney tissue culture and chick embryo tissue culture were infected with both the amoebae and type 3 poliovirus. There was no significant influence of the presence of one on the growth of the other. The infection of the cultures with amoebae did not make chick embryo cells susceptible to the poliomyelitis virus. The amoebic cysts also failed to support the growth of the poliomyelitis virus.

Staining reactions of the amoebae. The staining reactions of the amoebae were quite variable in both cystic and trophozoite stages. There was also great variability in staining coincident with intermedate stages between encystment and decystment.

Cysts. Typical cysts were very difficult to stain with any of the common stains such as azure eosin or hematoxylin (iron or alum), frequently remaining unstained. However, very good staining could be effected with the Bauer reaction(9) whereby the entire cyst stained red. They usually stained red or pink with the periodic acid-Schiff reaction, thereby indicating carbohydrate. They were yellow or unstained with the Van Gieson stain. The dinitrofluorobenzene H-acid method(10) for proteins stained the entire cyst red.

Trophozoites. With azure eosin, the karyosome and nuclear membrane stained blue and the nucleus faint blue. The cytoplasm stained a moderate irregular blue throughout because of numerous vacuoles and granules. Some of these granules stained with phosphotungstic acid hematoxylin (Fig. 3), Hale(9) periodic acid-Schiff (Fig. 4), dinitrofluorobenzene H-acid (Fig. 5) and oil red 0. It is apparent therefore that some of these granules contain protein, fat or carbohydrate. The oil red 0 method(9) also demonstrated fine droplets of fat on the trophozoite limiting cytoplasmic membrane.

Discussion. These observations reveal the adaptability of TC and its allied technics for the culture and study of certain amoebae. Shaffer and Sienkiewicz(11) and Shaffer(12) developed a method using chick embryo tissues suspended in TC nutrient fluid for cultivation of *Endamoeba histolytica.* Baernstein, Rees and Bartgis(13) grew *E. histoly-*

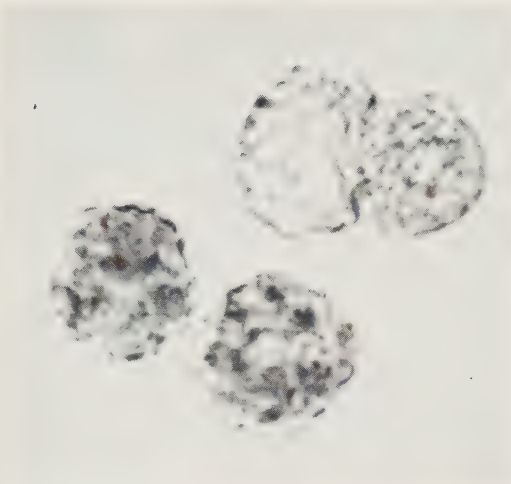

FIG. 3. Cyst and trophozoite-like forms of free living amoebae stained with phosphotungstic acid-hematoxylin. In stages that appear to be intermediate between cysts and trophozoites, some granules stain with phosphotungstic acid-hematoxylin and others do not. Cysts are usually unstained. × 1935.

tica in a suspension of fine particles from chick embryo homogenate. In the present study the amoebae grew very well in a TC system which was simple and free from bacteria. It is interesting to note that the amoebae failed to grow in Eagle's basal medium or Medium 199 which supported the growth of mammalian cells. The finding of amoebic cysts inside the cells suggests that further studies *in vitro* might throw new light on the interaction between the amoebae and the host cell. The cytolytic effect of the amoebae in HeLa cells offers another potential oncolytic agent for possible trial *in vivo*.

Summary. Two strains of apparently the same free living amoebae were found to be spontaneous contaminants in monkey kidney tissue culture. The TC technic proved to be a suitable method for cultivation of the amoebae in the absence of bacteria. Pure cultures of the amoeba were obtained in many cell lines. Both cysts and trophozoites were found in TC. In monkey kidney tissue culture, cysts were often found in the cytoplasm of the cells. One strain was subjected to 16 consecutive passages in monkey kidney tissue culture while the other had 12. The average CPE titer of the TC fluid was 10^4 to 10^5 per 1.0 ml. The cysts in the TC fluid survived at −50°C for eight months, although the titer was greatly reduced.

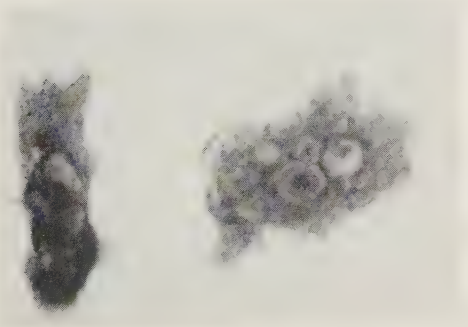

FIG. 4. Trophozoites of free living amoebae stained with periodic acid-Schiff and Mayer's hematoxylin. The cytoplasm is generally reactive for carbohydrate throughout. × 1650.

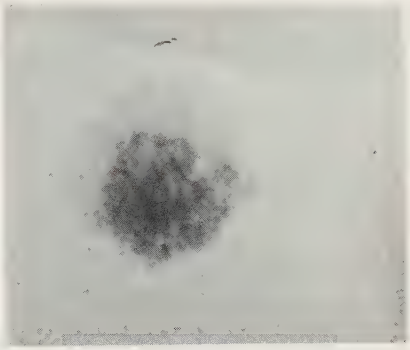

FIG. 5. Trophozoite of free living amoebae stained with dinitrofluorobenzene H-acid method for proteins. The cytoplasmic granules, karyosome and nucleus are strongly reactive. × 1800.

1. Dulbecco, R., and Vogt, M., *J. Exp. Med.,* 1954, v99, 167.

2. Youngner, J. S., PROC. SOC. EXP. BIOL. AND MED., 1954, v85, 202.

3. Warren, J., and Cutchins, E. C., *Virology,* 1957, v4, 297.

4. Takemoto, K. K., and Lerner, A. M., PROC. SOC. EXP. BIOL. AND MED., 1957, v94, 179.

5. Perry, V. P., Evans, V. J., Earle, W. R., Hyatt, G. W., and Bedell, W. C., *Am. J. Hyg.,* 1956, v63, 52.

6. Eagle, H., Oyama, V. I., and Levy, M., *Science,* 1956, v123, 845.

7. Eagle, H., Oyama, V. I., Levy, M., and Freeman, A., *J. Biol. Chem.,* 1957, v226, 191.

8. Melnick, J. L., and Riordan, J. T., PROC. SOC. EXP. BIOL. AND MED., 1952, v81, 208.

9. Lillie, R. D., Histopathologic technic and practical histochemistry, 2nd ed., Blakiston Co., N. Y. 1954.

10. Burstone, M. S., *J. Histochem. and Cytochem.,* 1955, v3, 32.

11. Shaffer, J. G., and Sienkiewicz, H. S., *Science,* 1952, v116, 306.

12. Shaffer, J. G., *Ann. N. Y. Acad. Sci.,* 1953, v56, Art. 5, 1033.

13. Baernstein, H. D., Rees, C. W., Bartgis, I. L., *Jour. Parasitology,* 1957, v43, 143.

Received August 26, 1957. P.S.E.B.M., 1957, v96.

Detection of Incomplete Enterobacterial Antibodies in Cord Blood by Means of Antiglobulin Hemagglutination Test.* (23516)

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The passive transfer of antibodies through the placenta from the mother to the fetus depends upon two main factors, namely, the structure and function of the placenta and the characteristics of the antibody. In some animal species, notably rodents, apes, and man, certain antibodies readily pass the placental barrier. In other species, such as the pig and ruminants, placental transmission does not take place, but antibodies are transferred to the offspring through colostrum. The role of the antibody molecule in the transmission through the placenta is illustrated by the fact that, for as yet poorly understood reasons, incomplete Rh antibodies pass the human placenta far more readily than complete antibodies of identical serologic specificity. Recently, it was shown by means of the indirect bacterial hemagglutination test, which is considerably more sensitive than the conventional bacterial agglutination method, that antibodies against the somatic (O) antigens of enteropathogenic *Escherichia coli* are not quantitatively transferred from mother to newborn infant. In a series of 26 cord blood specimens antibodies could be demonstrated in only 3, although the maternal serum of all contained such antibodies(1). Similarly, Stulberg and associates(2) found 77% of 45 cord serum specimens lacking in such antibodies. Further, when antibodies were present in cord blood, their titer was substantially lower than that of the corresponding maternal antibodies. The possibility was considered that incomplete antibodies to enterobacteriaceae may pass the human placenta, provided that the mother has such antibodies. Weil and Felsen(3) demonstrated incomplete *Shigella flexneri* agglutinins in approximately half of 30 human sera by combining the conventional bacterial agglutination method with the antiglobulin technic. To determine whether the hypothesis just mentioned is correct, maternal and cord serum specimens were examined by means of a hemagglutination-antiglobulin test for complete and incomplete enterobacterial antibodies.

Material and methods. Fifty paired maternal and cord blood specimens from healthy individuals were obtained through the cooperation of the Obstetrical Department of the Children's Hospital. The serum specimens were stored in the deep freeze until used. Data on blood groups and Rh antibodies were available. The hemagglutination test was carried out as described previously(4,5). As O antigens supernates of the following agar-grown cultures were employed: *E. coli,* serogroups 111 and 55, *Shigella flexneri* (2 freshly isolated and serologically different types), *S. sonnei, Salmonella paratyphi* B (group B), *S. montevideo* (group C_1), *S. muenchen-oregon* (group C_2), and *S. enteritidis* (group D). These supernates (refrigerated centrifuge) were prepared from heated (1 hour 100°C) suspensions and were employed in a dilution of 1:10. Erythrocytes obtained from blood donors (blood group O, Rh negative) were washed 3 times, treated with the antigens in a waterbath at 37°C for 30 minutes, and again washed three times. Serial two-fold dilutions of the sera (0.2 ml) were mixed with the modified red blood cell suspensions (0.2 ml). Non-

* Study aided by research grant from Natl. Inst. of Allergy, U. S. Public Health Service.

TABLE I. Enterobacterial Antibodies in 50 Cord Sera.

Method of antibody demonstration	% of positive sera								
	Escherichia coli		*Shigella flexneri*		*Shigella sonnei*	*Salmonella* group			
	111	55	a	b		B	C_1	C_2	D
Bacterial agglutination	0	0	0	0	0	0	0	0	0
Hemagglutination	18	6	14	78	6	12	52	20	10
Antiglobulin agglutination	30	28	24	14	22	30	18	46	30
Idem & hemagglutination	48	34	38	92	28	42	70	66	40

modified red blood cells were employed as controls to eliminate erroneous results due to blood group antibodies. After incubation for 30 minutes at 37°C in a waterbath the mixtures were centrifuged at 1000 rpm for 1 minute. The resulting hemagglutination was noted. After three washings antiglobulin (Coombs) serum (0.05 ml), procured on the open market, was then added to all tubes showing no hemagglutination. The tubes were centrifuged and the resulting hemagglutination, indicative of incomplete antibodies, was recorded.

Bacterial agglutination tests were carried out with the identical serum dilutions (0.5 ml) used in hemagglutination tests. Heated suspensions (0.5 ml) of the same cultures were used. The mixtures were incubated in a waterbath at 50°C for 18 hours, and the resulting bacterial agglutination was read after centrifugation for 5 minutes at 1500 rpm(6). As diluent phosphate hemagglutination buffer (Difco) was used in all tests.

Results. Fifty cord serum specimens from healthy infants were examined for O antibodies to 9 representatives of enterobacteriaceae by means of the conventional agglutination, enterobacterial hemagglutination, and antiglobulin hemagglutination methods. Sera were considered positive if antibodies were demonstrated in a dilution of 1:5 or higher. The antiglobulin hemagglutination test was regarded as positive only if the hemagglutination test itself was negative. The results are summarized in Table I.

It is seen from this Table that (1) none of the sera contained agglutinins, (2) from 6 to 78% yielded positive results in the hemagglutination test, and (3) an additional 14 to 46% were positive for incomplete antibodies in the antiglobulin hemagglutination test. By combining the hemagglutination and antiglobulin hemagglutination tests a total of 28 to 92% of the serum specimens yielded positive results. It is interesting to note that the percentage of positive sera differs markedly with different antigens, either because of actual differences in antibody content or because of differences in sensitivity of the antigens used. Particularly striking is the high percentage of sera containing antibodies to one of the *S. flexneri* strains.

Regarding the presence of incomplete enterobacterial antibodies in maternal and cord serum specimens the following observations were made. The antiglobulin serum raised the antibody titer of maternal sera only 2-fold in 17 instances and caused no increase in the remaining 33. In contrast, the same antiglobulin serum caused four-fold and greater increases in 22% and 8-fold and greater increases in 10% of 50 cord sera. In several serum specimens the titer was increased from 32 to 256 times. If one accepts a four-fold or greater enhancement in the antibody titer by the antiglobulin serum as evidence for incomplete enterobacterial antibodies, then, it may be concluded that such antibodies are present in certain cord sera and cannot be demonstrated by the same technic in the corresponding maternal sera. No difference was noted between cord sera with and without Rh antibodies.

A comparison of the maternal and cord serum titers obtained with both hemagglutination and antiglobulin hemagglutination tests revealed that with one single exception the maternal titers were higher than those of the cord sera. In the majority of instances the ratio was between 4:1 and 16:1.

In order to determine the specificity of the antiglobulin hemagglutination method, absorption experiments were carried out. Cord serum containing both *S. sonnei* and *S. flexneri* antibodies, demonstrable only by means of

antiglobulin serum, was absorbed with *S. sonnei* or *S. flexneri* antigens. Then, the absorbed and non-absorbed serum was titrated with both antigens in the antiglobulin hemagglutination test. It was found that absorption with *S. sonnei* or *S. flexneri* antigens had removed the homologous but not the heterologous antibodies. Identical results were obtained in experiments with other cord serum specimens. It is evident, therefore, that the antiglobulin hemagglutination method detects antibodies which become attached specifically to erythrocytes modified by the homologous enterobacterial antigen.

Discussion. The present study revealed that O antibodies to various enterobacteriaceae can be demonstrated in some human cord sera by means of the hemagglutination test and thus extends previous observations regarding hemagglutinins against enteropathogenic serotypes of *E. coli*. Since the conventional agglutination procedure yielded negative results, this observation confirms the greater sensitivity of the hemagglutination method. It is evident also that the sera differed markedly in their capacity to agglutinate red blood cells modified with different antigens. In the present series the high percentage of cord serum specimens with antibodies against one of the *S. flexneri* strains is particularly noteworthy. It remains to be determined whether these data reflect exclusively differences in antibody content or whether crude antigens prepared by the same method, as in the the present study, differ in sensitivity. In addition, the locality of the population may influence the antibody titers.

Incomplete enterobacterial antibodies are considered here to be present in cord sera if anti-human globulin serum enhances the serum titer obtained in the hemagglutination test at least 4-fold or produces a positive result when the latter test is negative. According to these criteria 22% of 50 cord sera contained incomplete enterobacterial antibodies against one or more antigens, but the corresponding maternal sera did not. These findings suggest the following hypothesis. Adult sera may contain both complete and incomplete enterobacterial antibodies, but the titer of the latter does not materially exceed that of the former; during pregnancy the incomplete antibodies pass the placental barrier more readily than do the complete antibodies. Additional studies are needed to determine whether the antiglobulin bacterial agglutination method of Weil and Felsen(3) measures the same antibodies as the antiglobulin hemagglutination test used in the present study. One must keep in mind that minor modifications of a serologic method may substantially enhance its sensitivity; for example, centrifugation renders the enterobacterial hemagglutination test considerably more sensitive. Therefore, the difference in titer between an agglutination procedure and the corresponding antiglobulin technic is not necessarily indicative of incomplete antibodies. Further, the possibility exists that the enterobacterial hemagglutination test does not solely measure bacterial agglutinins in a more sensitive manner; it actually may demonstrate more than one kind of antibody with identical serologic specificity. This possibility is suggested by the observation that no constant ratio exists between the titers determined by the two procedures. A similar suggestion was made by Ceppellini and De Gregorio and is based on comparative studies on *S. typhosa* antibody titrations by means of hemagglutination, hemolysis, hemagglutination in proteic media, and indirect Coombs test(7). Utilization of various serum fractions conceivably may aid in the elucidation of this moot question.

Summary. Examination of 50 paired maternal and cord sera for O antibodies against various enterobacteriaceae revealed the following results. 1. Agglutinins could not be demonstrated in the cord sera. 2. The hemagglutination test revealed the presence of antibodies in 6 to 78% of the cord sera depending upon the antigen used. 3. The antiglobulin hemagglutination test detected incomplete antibodies in an additional 14 to 46% of the cord sera. 4. The antiglobulin hemagglutination test enhanced the hemagglutinin titers of cord sera to a greater extent than those of maternal sera. 5. With one exception the maternal antibody titers were higher than those of the corresponding cord sera. 6. The specificity of the antiglobulin hemagglutination test was demonstrated by means of ab-

sorption experiments. 7. The placental transfer of enterobacterial antibodies from mother to fetus is discussed with particular reference to incomplete antibodies.

1. Neter, E., Westphal, O., Lüderitz, O., Gino, R. M., and Gorzynski, E. A., *Pediatrics,* 1955, v16, 801.

2. Stulberg, C. S., Zuelzer, W. W., and Page, R. H., *J. Immunol.,* 1956, v76, 281.

3. Weil, A. J., and Felsen, J., *ibid.,* 1955, v74, 488.

4. Neter, E., Gorzynski, E. A., Gino, R. M., Westphal, O., and Lüderitz, O., *Can. J. Microbiol.,* 1956, v2, 232.

5. Neter, E., Westphal, O., Lüderitz, O., and Gorzynski, E. A., *Ann. N. Y. Acad. Sci.,* 1956, v66, 141.

6. Braun, O. H., Seeliger, H., and Wagner, N., *Zschr. Kinderheilk.,* 1954, v75, 50.

7. Ceppellini, R., and De Gregorio, M., *Boll ist. sieroterap. milan.,* 1953, v32, 429.

Received August 29, 1957. P.S.E.B.M., 1957, v96.

Toxicological and Pathological Studies with 3,3-Dimethyl-1-Phenyltriazene: with Special Reference to Hepatic Damage.* (23517)

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During routine screening of compounds for anti-tumor effects, 3, 3-dimethyl-1-phenyltriazene was found to inhibit Sarcoma 180 in mice (1). The agent was also shown to be teratogenic in chick embryos(2) and to prolong the survival of mice bearing Leukemias 82 and 5471(3). Since this triazene was representative of a new series of compounds not previously known to be active against experimental tumors, it became of interest for possible clinical trial. For this reason pharmacological studies were undertaken in our laboratories.

Procedure. The kinds of animals used and the procedures employed have recently been described(4,5). The triazene, a liquid, was dissolved in peanut oil and 0.5 ml of the solution per 100 g of body weight was given intraperitoneally to mice and rats. For oral administration to rodents the agent was suspended in 0.9% NaCl containing 0.5% carboxymethylcellulose (CMC). For intravenous injections the compound was dissolved in propylene glycol while for intramuscular injection it was diluted with peanut oil. Dogs received the agent orally in gelatin capsules. LD_{50} values were calculated according to Litchfield and Wilcoxon(6) after administration of a sufficient number of doses, decreasing in series by a factor of ½, to obtain both 100 and 0% mortality during 14 days of observation. At least 10 mice, 6 rats, 2 cats, and 2 dogs were employed per dose.

Results. Toxicity in rodents. Table I is a summary of toxicity data. The compound was somewhat more toxic to mice and rats when given intraperitoneally than when given orally. Within 2 hours after 500 mg/kg intraperitoneally or 1000 mg/kg orally the righting reflexes of mice became impaired; a progressing depression ensued and death occurred between 3 and 20 hours. Following single doses of 250 mg/kg intraperitoneally or 500 mg/kg orally mice became depressed, dyspneic, and ataxic within 3 to 4 hours. A few died within the first day; most of the remainder between the second and eleventh days. The majority of those alive at 24 hours moved about in circles and their heads bobbed continuously from side to side. This syndrome, presumably neurologic in origin, was further characterized by the inability of the mice, when placed in water, to swim in a well-coordinated manner. The disturbance seemed similar to that seen in congenitally abnormal

* This investigation was supported in part by a grant from Natl. Cancer Inst., U.S.P.H.S., and by funds from the Elsa U. Pardee Fn.

TABLE I. Toxicity of 3,3-Dimethyl-1-phenyltriazene.

Species	Route of admin.	No. of successive daily doses	LD_{50},* mg/kg/day	19/20 confidence limits,* mg/kg/day	S*
Mice	Intraper.	1	200	160–250	1.30
	"	5	72	30–183	1.52
	Oral	1	290	200–420	1.82
Rats†	Intraper.	1	180	136–238	1.42
	"	5	71	64– 78	1.10
	Oral	1	180	333–608	1.35
	"	5	130	86–196	1.55
			Dose, mg/kg/day	No. dead/No. tested	Day of death
Cats	Intrav.	1	100	2/2	Immediate
			50	0/2	
			25	0/2	
	Intramusc.	1	200	2/2	1, 1
			100	1/2	3
			50	0/2	
Dogs	"	10‡	50	2/2	10, 18
			25	1/2	26
			12.5	0/2	
			6.25	0/2	
	Oral	10‡	50	1/2	35
			25	0/2	
			12.5	0/2	

* Calculated according to Litchfield and Wilcoxon(6). Animals were observed for at least 2 wk after the end of treatment.

† Carworth Farms Wistar (CFW).

‡ Maximum No. of doses given daily except weekends.

"waltzing" or "shaker" mice(7); the same syndrome has been induced by the administration of certain mono-2-chloroethylamines (8). Except for the absence of the neurological disturbance, the course of intoxication in rats given single lethal doses was like that seen in mice. When given on each of 5 successive days, the triazene caused progressive weight loss, ataxia, dyspnea, and depression in both mice and rats. At doses of 100 to 125 mg/kg/day intraperitoneally and 100-200 mg/kg/day orally deaths occurred between the sixth and eleventh days after the initiation of treatment. Pathological studies were not performed.

Pathological changes in rats. Lesions were studied in a group of 26 Carworth Farms Nelson (CFN) rats which received 100 mg/kg/day of the triazene in a maximum of 5 successive daily injections. Six animals were sacrificed at 2 days (24 hours after the second injection); at this time the rats appeared unkempt. On the fourth day (24 hours after the fourth injection) 6 additional animals were sacrificed; weight loss and "bloody" exudates about the nares were evident. Six rats were sacrificed on the seventh day (72 hours after the fifth injection); further weight loss had occurred and the rats appeared unkempt and had "bloody" nares. Four of the remaining 8 animals died on days 8, 16, 21, and 21, respectively. Control rats which had received injections of peanut oil were also sacrificed: at 2, 4, and 7 days. Significant hematological changes occurred during treatment (Table II). By the end of the second day reticulopenia and leukopenia were already evident; by the fourth day these changes were even greater and they were accompanied by a fall in the hematocrit. At the seventh day the reticulocyte count had returned to normal values but the leukopenia and anemia were still evident. On *gross* examination the following were noted in all treated animals: an "oily" abdominal ascites (present also in controls), semi-fluid or fluid bone marrow from the femoral shaft, and small thymi. At 4 and 7 days pallor of the inner cortex of the kidney was also prominent. Microscopic examination revealed lesions in a number of tis-

TABLE II. Weight and Blood Cells in Rats Treated with 3,3-Dimethyl-1-phenyltriazene. (Mean values ± stand. dev.)

Day of sacrifice	Wt change (%)	Reticulocytes (%)	Hematocrit (ml/100 ml)	Platelets (10^3 cells/mm^3)	WBC* (10^3 cells/mm^3)
Control†	+ 5 ± 5	3.1 ± 1.1	46.3 ± .9	437 ± 210	5.61 ± 1.7
2 days	− 3 ± 4	.6 ± .6	46.0 ± 3.3	506 ± 127	1.04 ± .5
4	−17 ± 3	.01 ± .04	42.0 ± 4.3	602 ± 172	.11 ± .1
7	−29 ± 3	4.0 ± 3.6	29.0 ± 5.4	503 ± 160	.05 ± .1

* White blood cells. † Avg of 12 animals, 4 at each of days 2, 4, and 7.

sues as early as 2 days after initiation of treatment. These progressed in severity and in all 6 animals sacrificed at 7 days they were as follows: the lymphocytic elements of spleen, thymus, and nodes were markedly depleted; the red pulp of the spleen was congested and the extra-medullary hematopoietic cells were reduced in number; the sternal bone marrow showed a 90 per cent loss of nucleated elements; spermatogenic arrest was observed in some of the testicular tubules and this was associated with multinucleated giant cells; in the kidneys there was necrosis in the distal portion of the proximal convoluted tubules as well as a mild membranous glomerulonephritis; a peripancreatic inflammatory reaction was present. The livers appeared normal at 2 and 4 days but at 7 days showed a loss of cytoplasmic basophilia and a slight enlargement of hepatic cells in central zones. Hepatic necrosis was not evident. Finally, at 2 and at 4 days the mucosa of the small bowel had some atypia manifested by nuclear swelling and loss of polarity of the inner mucosal glands; by 7 days the small intestine showed no abnormalities.

Toxicity in cats. Table I presents the toxicity data for triazene in cats. The pair given 100 mg/kg intravenously showed immediate respiratory arrest. Cats given 200 mg/kg intramuscularly died unobserved overnight; one was dyspneic for several hours after injection while the other became ataxic after 4 hours and developed paralysis of the hind legs. Of the pair given 100 mg/kg intramuscularly one developed ataxia of the hind legs by 10 hours. This was no longer present at 24 hours and the cat survived. The other animal died at 3 days; it was markedly ataxic, dyspneic, and cyanotic on the day after injection and exhibited episodes of tonic and clonic convulsions. No remarkable changes were noted in animals receiving 50 mg/kg intravenously or intramuscularly. Pathological studies were not performed.

Pathological changes in dogs. The toxicity data in dogs are given in Table I. In the 8 animals given 25 or 50 mg/kg/day anorexia, weight loss, and emesis became evident during the first week of treatment. Six of this group became jaundiced during treatment or within 1 week thereafter. Dilute urine (specific gravity $\leqq$ 1.015) was also observed in 6 of the 8 animals. Body temperatures remained relatively normal throughout the course of fatal intoxications. To determine the lesions associated with intoxication 4 additional dogs were given the doses described in Table III and sacrificed at different times during or after treatment. As seen in Table III, each animal lost weight during treatment. Dogs 2 and 4 showed a decrease in the hematocrits without concomitant decrease in reticulocyte counts. Lymphocytopenia became evident in 3 dogs but there were no consistent changes in the counts of neutrophilic granulocytes. Thrombocytopenia developed in dogs 3 and 4 though blood clotting time was not significantly affected. In dogs 3 and 4 there developed prolonged prothrombin times and severe deficiencies in the fibrinogen content of plasma. This evidence of hepatic insufficiency was associated with hyperbilirubinemia and increased sulfobromophthalein (BSP) retention. In addition the activity of plasma alkaline phosphatase rose in each animal and especially in dog 4. The total serum protein decreased in all animals. In other biochemical analyses, which are not recorded in Table III, plasma non-protein nitrogen and blood glucose remained unchanged while decreases were observed in plasma chloride to as low as 70 meq/l at day 15 in dog 3.

At autopsy the tissues of the 3 dogs given

TABLE III. Hematological and Biochemical Data from Dogs Treated with 3,3-Dimethyl-1-phenyltriazene.

Dog	Day	Wt (kg)	Herit (ml/100 ml)	Retic (%)	Leyte (10^3 cells/mm^3)	Pltlt	Prot (g %)	Prothb time (sec.)	Blrbn	BSP	Alk ptase	Fbgn
									(mg %)			
1	50 mg/kg × 5, IM											
	0	14.8	52.5	tr	2.6	208	5.9	13.9	.3	.2	.5	
	4	12.9	53	.3	.1		5.3	13.8	1.0	1.4	1.5	
	7 *S*	12.7	52	.3	.1	120	5.1	14.7	8.3	3.5	3.3	
2	50 mg/kg × 7, PO											
	0	10.3	53	1.0	3.7	192	5.5	13.4	.2	.3	2.2	
	7	9.1	41.5	.6	.3	174	5.1	10.3	.5	1.1	4.8	
	9 *S*	8.7	39	1.4	.1	180	4.4	10.9	.8	1.6	9.8	
3	25 mg/kg × 10, IM											
	0	14.8	54	.1	3.0	158	5.8	13.9	.2	.2	1.0	233
	8	13.1	53	.2	1.5	280	5.1	20.5	.7	2.0	5.0	218
	15 *S**	12.1	51	1.2	.7	46	2.9	45	11.8	4.0	6.7	†
4	25 mg/kg × 10, IM											
	0	11.1	52	.2	.8	176	5.0	12.3	.2	.2	.8	234
	8	9.9	45	.8	.5	278	5.0	12.6	.7	.8	2.3	363
	15	8.7	46	2.6	.6	76	4.4	17.9	4.5	3.0	6.2	226
	18	8.5						32				
	22	8.2	43	1.5	.9	10	4.2	22.9	3.9	2.7	19.9	110
	30	8.5	49	.5	.6	46	4.4	19.6	1.7	3.2	20.3	†
	32 *S*	8.7	45	.3	.9	42		19.8	1.2	2.6	19.8	180

Abbreviations: Alk ptase, plasma alkaline phosphatase; Blrbn, total plasma bilirubin; BSP, sulfobromophthalein retention in blood; Fbgn, plasma fibrinogen; Herit, hematocrit; Leyte, lymphocytes; Pltlt, platelets; Prot, total plasma protein; Prothb time, one-stage prothrombin; Retic, reticulocytes; IM, intramusc.; PO, oral.

* Dog 3 was moribund at sacrifice.

† The oxalated plasma failed to clot upon addition of $CaCl_2$ and thrombin.

the triazene intramuscularly were jaundiced and the lobular markings of their livers were accentuated. The liver of dog 3 appeared to be fatty. This animal as well as dog 4 had clear yellow ascitic fluid in the abdomen. Except for minute hemorrhages in the pylorus of the stomach and bloody mucus in the ileum of dog 2, which had received the oral dose, there were no other noteworthy gross findings. On *microscopic* examination the most prominent changes were found in the livers of all 4 dogs. These are described in detail because the lesions varied considerably in the different individuals and because the hepatotoxic effects appeared to be novel. Dog 1 showed damage in the central zone affecting approximately one-third of the lobule. In the involved portions the majority of the hepatic cells were small and there was disorganization of their cord-like arrangement. Loss of cell boundaries was frequent and cytoplasmic vacuolization was evident. A few deeply eosinophilic hepatic cells were present intermixed with polymorphonuclear leukocytes. Canaliculi showed bile-plugging in all portions of the lobule but most prominently in central zones. Some medium-sized bile ducts contained inspissated bile and in these there was some loss of nuclear polarity in the epithelium. The liver of dog 2 had changes that were similar except for involvement of only about one-fourth of the lobule and less prominent plugging of the bile canaliculi. On the other hand more diffuse and extensive effects were observed in the livers of dogs 3 and 4, which were sacrificed at later times, 15 and 34 days, respectively. It is interesting to recall that the plasma prothrombin times became markedly elevated in dogs 3 and 4 after the end of the first week of treatment (Table III). Nearly all liver cells were larger and more varied in shape than normal. The cord-like arrangement was lost resulting in disorganization of the lobular pattern. In the central zones the cytoplasm of most liver cells was finely vacuolated while in peri-portal regions it was deeply eosinophilic. Many cells contained enlarged and irregular nuclei and nu-

cleoli, prominently in the periphery of the lobule. Necrotic cells containing eccentric nuclei and dark pink cytoplasm were scattered throughout the liver; some of these were accumulated in small nests. In dog 3 there was plugging of canaliculi and inspissation of bile with loss of polarity in the epithelium of the medium ducts. No inflammatory reaction was observed. In dog 4 although canalicular stasis was minimal, bile-laden macrophages were present around the central veins intermixed with red cells, polymorphonuclear leukocytes, and lymphocytes.

The other lesions seen are briefly recorded. The bone marrows of all 4 dogs were congested; but only in dog 1 was there found a slight diminution in the number of nucleated elements. In dogs 3 and 4 the sinuses of the lymph nodes contained red cells and acute ulcers were present in the tonsils. Recent ulcers were also found in the buccal mucosa and esophagus of dog 3. In the same animal small hemorrhages were present in the adrenals and colon and the small intestines were congested; interstitial edema of the pancreas was noted with occasional inflammatory cells and some disorganization of the acinar pattern; spermatogenic arrest and numerous giant cells were seen in the testes. In dog 4 necrotic cells were found in the ovarian follicles; a membranous glomerulonephritis was present in its kidneys as well as a solitary focus of acute pyelonephritis.

Discussion. The present study has revealed the major pathologic changes induced by 3, 3-dimethyl-1-phenyltriazene in rats and dogs. The peripheral blood, the bone marrow, and the kidney were the primary sites of abnormality in the rat. In dogs the principal pathologic changes were confined to the liver. These included abnormalities in the size and shape of hepatic cells, necrosis of hepatic cells, disorganization of the lobular pattern, and intrahepatic biliary stasis. Biochemical changes indicative of hepatic dysfunction were associated with the liver lesion, namely, increases in the one-stage prothrombin time, hyperbilirubinemia, elevations in the retention of sulfobromophthalein and in the activity of serum alkaline phosphatase, and decreases in the fibrinogen content of plasma.

The contrast in the sites of action in the two species was probably not an absolute one; for hematologic changes such as thrombocytopenia and a slight hypoplasia of bone marrow were noted in a few dogs and a membranous glomerulonephritis was seen in one. Conversely intoxicated rats evidenced minor alterations in the hepatic parenchyma.

The mode of action of the triazene may be related to certain of its chemical properties. Diazoamino compounds are cleaved by acid catalysis to amines and diazonium ions; in the present instance the products would be dimethylamine and benzenediazonium cations. The amine probably has little effect in the amounts formed; but the diazonium moiety could either react directly with cellular constituents or undergo further toxic transformations. For example, hydrolysis would result in phenol while mild reduction would give rise to phenylhydrazine(9). At physiologic pH the cleavage of the triazene would probably be slow, thereby permitting distribution of intact molecules throughout the tissues. If their distribution were non-uniform, varying amounts of toxic moieties would be formed at different sites resulting in selective tissue damage.

In spite of these likely possibilities it has not been possible to explain precisely all of the effects of the present agent in terms of the known properties of the toxic products just mentioned. For example, the anemia in rats and the reticulocytosis and jaundice in dogs seemed suggestive of the hemolytic effects of phenylhydrazine(10). Since the formation of damaged erythrocytes by simple benzene derivatives as well as by phenylhydrazine may be characterized by the presence of Heinz bodies, these abnormalities were sought in the red cells of a pair of dogs given 50 mg/kg/day of the triazene for 5 days (a lethal dose). Smears of daily blood samples, stained by Webster's technic (11), failed to reveal any specific red cell alteration. Since phenol is acutely toxic to the central nervous system and produces convulsions in mice, rats, and cats(12), its formation seemed suggested in the "neurological" syndrome caused by the present agent in mice. Nevertheless, the responses of mice to equimolar, lethal,

single doses of phenol given intraperitoneally were readily distinguished from those caused by the triazene. Moreover, the latter substance was not convulsant in rats or cats.

On the other hand the hepatotoxic and nephrotoxic effects of the triazene may be more readily related to possible transformations *in vivo;* for both phenol(13) and phenylhydrazine(14) have been reported to cause liver and kidney damage in laboratory animals. In this connection it is of interest to note that Kirby (15) has found lesions in liver, spleen, and kidney following the administration of 1,3-diphenyltriazene. This author suggested that the hepatic effects were not due to an *in vivo* rearrangement to *p*-aminodiazobenzene since the latter produces a lesion different from that caused by diphenyltriazene. It is conceivable that the diphenyl derivative may undergo cleavage *in vivo,* as suggested in the case of the agent used herein, with the formation of benzenediazonium cations and aniline.

Summary. The toxicity of 3,3-dimethyl-1-phenyltriazene has been described in various laboratory mammals. The agent produced a "neurological" disturbance in mice resembling that seen in the congenitally abnormal "waltzing" or "shaker" strains. In rats lesions were prominent in hematopoietic tissues and in the kidney. In dogs an unusual hepatic lesion was induced which was associated with a variety of disturbances in liver function. The effects of the compound were discussed with respect to possible transformation *in vivo* into phenyldiazonium and various derivatives of the latter substance. Hepatic insufficiency, renal damage, and hematopoietic depression should be anticipated in clinical trials with the agent.

The 3,3-dimethyl-1-phenyltriazene was kindly supplied by Dr. C. S. Rondestvedt, Jr., University of Michigan, Ann Arbor, and by Merck & Co., Rahway, N. J.

1. Clarke, D. A., Barclay, R. K., Stock, C. C., and Rondestvedt, C. S., Jr., Proc. Soc. Exp. Biol. and Med., 1955, v90, 484.

2. Dagg, C. P., Karnofsky, D. A., Stock, C. C., Lacon, C. R., and Roddy, J., *ibid.,* 1955, v90, 489.

3. Burchenal, J. H., Dagg, M. K., Beyer, M., and Stock, C. C., *ibid.,* 1956, v91, 398.

4. Scholler, J., Philips, F. S., and Sternberg, S. S., *ibid.,* 1956, v93, 398.

5. Philips, F. S., Sternberg, S. S., Hamilton, L. D., and Clarke, D. A., *Cancer,* 1956, v9, 1092.

6. Litchfield, J. T., Jr., and Wilcoxon, F., *J. Pharmacol. and Exp. Therap.,* 1949, v95, 99.

7. Grüneberg, H., *The Genetics of the Mouse,* 1952, Martinus Nijhoff, The Hague.

8. Goldin, A., Noe, H. A., Landing, B. H., Shapiro, D. M., and Goldberg, B., *J. Pharmacol. and Exp. Therap.,* 1948, v94, 249.

9. Saunders, K. H., *The Aromatic Diazo-Compounds and their Technical Applications,* 1949, Longmans, Green & Co., New York.

10. Wintrobe, M. M., *Clinical Hematology,* 3rd Edition, 1942, Lea & Febiger, Philadelphia.

11. Webster, S. H., Liljegren, E. J., and Zimmer, D. J., *Stain Technology,* 1948, v23, 97.

12. Deichmann, W. B., and Witherup, S., *J. Pharmacol. and Exp. Therap.,* 1944, v80, 233.

13. Deichmann, W., Kitzmiller, K. V., and Witherup, S., *Am. J. Clin. Path.,* 1944, v14, 273.

14. Bodansky, M., Marr, W. L., and Brindley, P., *Am. J. Clin. Path.,* 1932, v2, 391.

15. Kirby, A. H. M., *Cancer Research,* 1947, v7, 263.

Received September 3, 1957. P.S.E.B.M., 1957, v96.

Influence of Dietary Potassium on Potassium-42, Rubidium-86 and Cesium-134 Retention in the Rat.* (23518)

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MacLeod and Snell(1) reported that cesium, potassium and rubidium behaved similarly in the nutrition of lactic acid bacteria. These elements are known to enter the solute complex, participating in ion antagonism, osmosis, permeability regulation, maintenance of the colloidal state and similar physiological phenomena(2). Mraz *et al.*(3) observed an increase in excretion of cesium-134 by the rat when dietary potassium was increased. Mraz and Patrick(4) demonstrated that the absence of dietary potassium in a casein-cornstarch diet decreased urinary and fecal excretion of cesium-134, potassium-42 and rubidium-86. Miller(5,6) observed that the minimum potassium requirement for rats lies somewhere between 0.55 and 1.44 g per kilo of diet. This study was initiated to ascertain the feasibility of utilizing potassium-42, rubidium-86 and cesium-134 as indicators of potassium requirement in the rat.

Methods and materials. The basal diet used in these studies is the casein-cornstarch diet of Mraz and Patrick(7) minus the potassium chloride. Potassium in the chloride form was substituted for part of the cornstarch in the basal diet. Four albino rats (Rockland strain) were used per diet in each of the experiments. The rats in the potassium-42 trial were fed their respective diets for 14 days and those in the rubidium-86 and cesium-134 trials for 7 days before administration of the radionuclides. Five microcuries of cesium-134 or 35 microcuries of rubidium-86 were administered in the cesium or rubidium trials and a 72-hour balance trial made. The short half-life of potassium-42, 12.5 hr as compared to 2.3 years for cesium-134 and 18.6 days for rubidium-86, necessitated the use of 200 microcuries of the nuclide and only a 48-hour balance trial. The rats were sacrificed at the end of the balance trials. All nuclides were subcutaneously administered in the chloride form. Cesium-134 was used at tracer levels, while the potassium-42 and rubidium-86 dosages contained 26 mg potassium and 8.2 mg rubidium, respectively. Feces and urine samples were collected every 24 hours after administration of the nuclides. Excreta were digested with nitric acid and aliquots counted in a scintillation well-type counter. In the interest of ease of recording and reading, all data have been reported utilizing a maximum of three significant figures and are not indicative of sensitivity of the experiments. Least significant differences at the 5% level of confidence were determined and used as criteria for reporting significance between tissues and excreta as attributable to treatments.

In each experiment 8 groups of rats were fed either 0, 0.5, 0.075, 0.1, 0.15, 0.2, 0.3 or 0.4% potassium. At initiation of the trials, average weights of the rats were 100, 80, and 60 g for the potassium-42, rubidium-86 and cesium-134 trials, respectively.

Results. It may be noted (Table I) that rats fed diets containing 0.15 or more per cent potassium retained significantly less potas-

TABLE I. Potassium-42 Content of Tissues and Excreta as Influenced by Varying Levels of Potassium.

Dietary K (%)	Wt gain (14 day) (g)	Tissue* (%/g)			Excretion* (48 hr)
		Muscle	Kidney	Liver	
.00	−2	1.34	1.10	1.38	.8
.05	18	1.15	.89	1.06	.6
.075	24	1.21	.86	1.05	3.4
.10	32	1.02	.81	.89	5.1
.15	56	.75	.53	.70	8.0
.20	55	.79	.59	.69	10.7
.30	58	.73	.54	.65	10.1
.40	39	.84	.63	.75	15.2
L.S.D.†		.24	.23	.23	6.0

* Expressed as % of subcut. administered dose.
† Least significant difference between means at 5% level of confidence.

* This manuscript is published with permission of Director of Univ. of Tennessee, Agric. Exp. Station, Knoxville. Radioactive materials used in this work were obtained from Oak Ridge Natl. Laboratory on allocation from U. S. Atomic Energy Com.

sium-42 in their tissue than did those fed diets containing 0.075 or less per cent potassium. Significantly more potassium-42 was excreted by rats fed diets containing at least 0.15% potassium than by those fed diets containing 0.05 or less per cent potassium.

Significant differences in rubidium-86 uptake by tissues were observed (Table II) but the level of potassium at which significance was found varied between tissues. The rats fed 0.15% potassium excreted significantly more rubidium-86 than did those fed lower levels. Each increment of dietary potassium fed beyond 0.15% induced a significant increase in rubidium-86 excretion.

Tissue uptake of cesium-134 (Table III) proved to be quite variable with no sharp trends being apparent. Excretion of cesium-134, however, showed more uniform trends with the first significant difference in cesium-134 excretion occurring at 0.15% potassium and the next at 0.20% after which it levels off.

Growth rate in all 3 experiments began to plateau at 0.15% potassium. The tissue uptake of radionuclides on the various potassium levels is believed to be partially a reflection of body weight of the rats at time of dosing. Since the rats fed potassium deficient diets did not grow as well as those on potassium adequate diets, their body weights were lower at the end of the feeding period. The smaller the rat, therefore, the higher the concentration of radionuclides per gram of tissue, even if excretion of the nuclides remained constant.

TABLE II. Rubidium-86 Content of Tissues and Excreta as Influenced by Varying Levels of Potassium.

Dietary K (%)	Wt gain (7 day) (g)	Tissue* (%/g)			Excretion* (72 hr)
		Muscle	Kidney	Liver	
.00	3	1.66	2.15	2.42	1.2
.05	15	1.66	1.80	2.06	1.7
.075	17	1.47	1.55	2.13	1.6
.10	22	1.42	1.58	1.67	2.1
.15	26	1.32	1.45	1.74	3.6
.20	29	1.18	1.00	1.39	4.8
.30	27	1.24	1.37	1.50	6.9
.40	31	1.18	1.37	1.37	9.8
L.S.D.†		.35	.35	.41	1.2

* Expressed as % of subcut. administered dose.
† Least significant difference between means at 5% level of confidence.

TABLE III. Cesium-134 Content of Tissues and Excreta as Influenced by Varying Levels of Potassium.

Dietary K (%)	Wt gain (7 day) (g)	Tissue* (%/g)			Excretion* (72 hr)
		Muscle	Kidney	Liver	
.00	2	2.79	3.27	1.18	4.1
.05	8	2.53	3.64	1.26	7.0
.075	15	2.90	3.10	1.40	7.9
.10	19	2.32	3.47	1.11	8.1
.15	26	2.09	2.24	1.03	14.3
.20	27	1.93	2.38	.93	19.0
.30	21	1.88	2.21	.97	21.1
.40	27	1.71	1.85	.84	21.1
L.S.D.†		N.S.	1.18	N.S.	4.8

* Expressed as % of subcut. administered dose.
† Least significant difference between means at 5% level of confidence.

Soft tissue-seeking radionuclides such as cesium-134, potassium-42 and rubidium-86 show promise as indicators of dietary potassium deficiency in rats. If one postulates that these radionuclides partially substitute for potassium in a deficient animal, the results may be interpreted to indicate that the dietary requirement lies between 0.075 and 0.15% potassium, probably between 0.1 and 0.15%. Of course, impaired metabolism due to potassium deficiency may be the factor influencing excretion of these radionuclides, but the interpretation of data would still be valid. The growth data would also tend to support this supposition. More investigations will have to be conducted, however, before the radionuclide method can be quantitatively used for determination of animal requirements.

Summary. An increase in dietary potassium has been shown to increase excretion of cesium-134, potassium-42 and rubidium-86. Interpretation of the first significant increase in excretion of these radionuclides as being the point at which dietary potassium is adequate would place the potassium requirement of the rat between 0.075 and 0.15%, probably between 0.1 and 0.15%. This is supported by the growth data.

This work was completed under contract between Univ. of Tennessee, College of Agric. and Atomic Energy Com.

1. MacLeod, R. A., and Snell, E. E., *J. Bact.*, 1950, v59, 783.

2. Hober, R., *Physical Chemistry of Cells and Tissues,* 1945, Blakiston, Phila., Pa.

3. Mraz, F. R., LeNoir, M., Pinajian, J., and Patrick, H., *Arch. Biochem. and Biophys.,* 1957, v66, 177.

4. Mraz, F. R., and Patrick, H., PROC. SOC. EXP. BIOL. AND MED., 1957, v94, 409.

5. Miller, H. G., *J. Biol. Chem.,* 1923, v55, 61.

6. ———, *ibid.,* 1926, v70, 587.

7. Mraz, F. R., and Patrick, H., *J. Nutrition,* 1957, v61, 535.

Received September 3, 1957. P.S.E.B.M., 1957, v96.

Concurrent Concentrations of Human Salivary Buffer Components in Serum and Saliva.* (23519)

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The buffer systems in human saliva constitute a major natural defense mechanism against dental decay. Quantitatively, the buffer concentration of saliva varies with glandular activity, stimulated saliva having an appreciably higher buffer level than unstimulated saliva(1).

Numerous investigators have shown that the salivary buffer capacity of caries-resistant persons exceeds that of caries-active individuals(2,3,4,5). In a given subject under physiologic conditions, the buffer concentration of saliva varies only slightly from hour to hour and from time to time. It has been stated that the buffering power of saliva is directly dependent upon that of blood(6). Experiments in this laboratory have shown that metabolic acidosis depresses the salivary buffer capacity and that a similar reaction follows the administration of 2-acetylamino-1,3,4-thiadiazole-5-sulfonamide (Diamox) a carbonic anhydrase inhibitor(7).

Materials and methods. Specimens of venous blood and paraffin stimulated saliva were obtained simultaneously from 34 patients of the Nutrition Clinic, Hillman Hospital. The samples were collected in sterile tubes under paraffin oil in the morning before breakfast and before brushing the teeth. The serum was drawn off after centrifugation and saved for analysis under paraffin oil. Neither the paraffin blocks which served as secretory stimulants nor the paraffin oil contained detectable amounts of any of the constituents tested. The buffer capacities of samples of serum and saliva through the pH range 7 to 6 were determined by the method of Dreizen, Mann, Cline and Spies(8) using 0.1 N lactic acid for the development of the titration curves. The sodium and potassium content of each sample was assayed with a Perkin-Elmer Model 52-A flame photometer by the procedure of Bills, McDonald, Niedermeier and Schwartz applying the internal standard technic(9). The serum and saliva concentrations of bicarbonate, phosphate and protein were measured by the methods of Van Slyke and Neill(10), Fiske and SubbaRow(11) and Wolfson, Cohn, Calvary and Ichiba(12) respectively. The values for each constituent were converted into milliequivalents per liter using the factors described in a previous publication(1). The data were then subjected to statistical analysis.

Results. The ranges and mean concentrations of the anionic and cationic constituents of the buffer systems measured in the serum and saliva of the 34 patients are shown in Table I. The ranges in saliva exceeded those in serum for each ion except protein. Sodium had the widest range in saliva and bicarbonate in serum. The mean levels in milliequivalents/liter were: Na^+, 32.80 in saliva and 145.72 in serum; K^+, 20.40 in saliva and 5.10 in serum; HCO_3^-, 15.60 in saliva and 27.04 in

* Northwestern University studies in nutrition at Jefferson-Hillman Hospital, Birmingham, Ala. These studies were supported by grants from the Avondale Educational and Charitable Fn., Metropolitan Fn. of Atlanta, and Republic Steel Corp.

TABLE I. Comparison of Buffer Components of Serum and Saliva in 34 Patients.

Components	Mean		σ*		r†	σ_r‡
	Saliva	Serum	Saliva	Serum		
Sodium (meq/l)	32.80	145.72	17.44	3.03	.255	.160
Potassium "	20.40	5.10	4.04	.59	.112	.157
Bicarbonate "	15.60	27.04	2.13	1.95	.035	.171
Phosphate "	10.17	2.73	2.97	.71	−.019	.171
Protein "	.94	16.19	.33	.70	−.208	.164
Buffer capacity (ml 0.1 N lactic acid)	.68	1.14	.17	.16	.392	.145

* σ = Stand. dev. † r = Coef. of correlation. ‡ σ_r = Stand. error of coef. of correlation.

serum; $HPO_4^=$, 10.17 in saliva and 2.73 in serum; and protein$^-$, 0.94 in saliva and 16.19 in serum. The mean buffer capacities through pH range 7 to 6 were 0.68 and 1.14 ml 0.1 N lactic acid for saliva and serum respectively. In each of the 34 patients the saliva potassium and phosphate concentrations exceeded the serum levels whereas the serum sodium, bicarbonate and protein values surpassed those of saliva. The buffer capacity of serum through pH range 7 to 6 was greater than that of stimulated saliva in the same pH range in every patient. The specific values are shown in the scattergram contained in Fig. 1.

The coefficients of correlation (r) developed from a comparison of the concentrations of each of the five buffer components in serum and saliva were: 0.255 for Na^+, 0.112 for K^+, 0.035 for HCO_3^-, −0.019 for $HPO_4^=$, and −0.208 for protein$^-$. The correlation coefficient for the buffer capacity of serum and saliva in the pH range 7 to 6 was 0.392. In no instance did the coefficients of correlation surpass the standard error of the coefficient of correlation (σ_r) by the more than 3 times required for statistical significance.

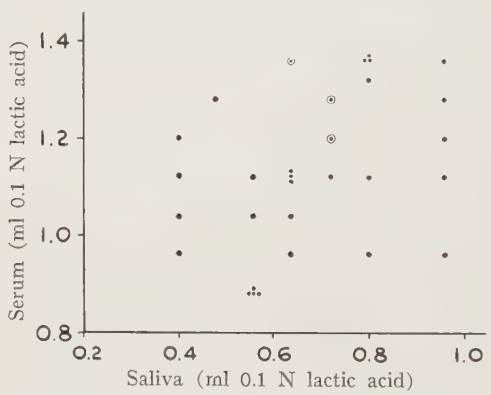

FIG. 1. The buffer capacities of serum and saliva in 34 patients (pH range 7.0-6.0).

Discussion. The most important buffer systems in blood and saliva are salts of weak acids and strong bases. In each, the main constituents are sodium, potassium, bicarbonate, phosphate and protein. At the normal pH of blood, the greatest proportion of buffer action in serum is contributed by bicarbonate with protein next in importance (13); at the pH of stimulated saliva, bicarbonate is the principal buffer with minor contributions from the phosphates(14,15). In each of the 34 patients the concentration of bicarbonate in serum exceeded that of stimulated saliva when the samples were collected concurrently. The difference in the bicarbonate content was reflected in the uniformly greater buffer capacity of serum in contrast to saliva in the pH range 7 to 6. This range includes the pK_1 of bicarbonate, the level at which the buffer action of this system operates at maximum efficiency.

The lack of statistically significant correlation between the buffer capacities of stimulated saliva and serum indicates that the composition and level of salivary buffers is dependent upon the biochemical activity and selectivity of the salivary glands. By such selective action on blood electrolytes, the salivary glands produce a secretion which is hypotonic to serum, a property unique among the major glandular secretions of the alimentary tract. The recent demonstration that oral microbial flora and saliva sediment contain buffer substances(16) indicates that both glandular and extraglandular sources contribute to the total buffering capacity of saliva.

Summary. The present study is concerned with a comparison of the buffer components and buffer capacities (pH range 7 to 6) of

paraffin stimulated whole saliva and blood serum in 34 patients from whom samples were collected simultaneously. 1) In every patient, the buffer capacity of serum exceeded that of saliva in the pH range tested; in each, saliva contained higher concentrations of potassium and phosphate than serum, and serum contained greater concentrations of sodium, bicarbonate and protein than saliva. 2) There was no statistically significant correlation between the buffer capacities and the main buffer constituents of serum and stimulated whole saliva. 3) Thus, the quantitative composition of the salivary buffers which act to protect against dental caries is determined primarily by glandular activity and not by simple ultrafiltration.

1. Dreizen, S., Reed, A. I., Niedermeier, W., and Spies, T. D., *J. D. Res.*, 1953, v32, 497.

2. Mann, A. W., Dreizen, S., Spies, T. D., and Hunt, F. M., *J. Am. Dent. Assn.*, 1947, v34, 244.

3. Ericsson, Y., *Acta Odont. Scandinav.*, 1949, v8, Suppl. 3, 1.

4. Turner, N. C., and Anders, J. T., *J. D. Res.*, 1956, v35, 385.

5. Muracciole, J. C., *idem.*, 1955, v34, 387.

6. Hafer, H., *Zahnartz, Praxis,* 1956, v7, 3.

7. Niedermeier, W., Stone, R. E., Dreizen, S., and Spies, T. D., PROC. SOC. EXP. BIOL. AND MED., 1955, v88, 273.

8. Dreizen, S., Mann, A. W., Cline, J. K., and Spies, T. D., *J. D. Res.*, 1946, v25, 213.

9. Bills, C. E., McDonald, F. G., Niedermeier, W., and Schwartz, M. C., *Anal. Chem.*, 1949, v21, 1076.

10. Van Slyke, D. D., and Neill, J. M., *J. Biol. Chem.*, 1924, v61, 523.

11. Fiske, C. M., and SubbaRow, Y., *ibid.*, 1925, v66, 375.

12. Wolfson, W. Q., Cohn, C., Calvary, E., and Ichiba, F., *J. Clin. Path.*, 1948, v18, 723.

13. Viikari, S. J., Harjola, P., and Maamies, T., *Scandinav. J. Clin. and Lab. Investigation,* 1954, v6, 122.

14. Wah Leung, S., *J. D. Res.*, 1951, v30, 403.

15. Sellman, S., *Acta Odont. Scandinav.*, 1949, v8, 244.

16. Lilienthal, B., *Oral Surg., Oral Med., Oral Path.*, 1955, v8, 828.

Received September 27, 1957. P.S.E.B.M., 1957, v96.

Role of Enzymatic Adaptation in Production of Experimental Alkaptonuria.* (23520)

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Papageorge and Lewis reported that rats maintained on high phenylalanine diets began, after a lag period of as long as 3 weeks, to excrete homogentisate in their urine(1). After this demonstration it was no longer possible to consider, as Dakin had, that homogentisate was an abnormal metabolite formed only by individuals with the rare hereditary disease of alkaptonuria. It has since been well established that homogentisate is normally formed in mammalian liver in the course of the metabolism of tyrosine and phenylalanine (2). Biochemical genetics has suggested a mechanism for hereditary alkaptonuria: the inactivity of the genetically controlled enzyme that degrades homogentisate. No mechanism has been suggested for the dietary alkaptonuria, but the lag period before its appearance suggests that some metabolic adaptation (11) must occur. In the present studies, the activities of the several enzymes acting successively to form and degrade homogentisate have been measured during the production of experimental alkaptonuria in rats. It was expected that adaptive changes in the amount of one enzyme relative to another would occur which would account for the accumulation of homogentisate. This expectation was con-

* This investigation was supported by grant from the Natl. Inst. of Arthritis and Metabolic Diseases, U. S. P. H. S., and by U. S. Atomic Energy Com. contract with the New England Deaconess Hospital.

firmed. Tyrosine feeding was used in the present experiments for economy of materials and assays, since one less enzyme step was involved in the production of homogentisate from tyrosine than from phenylalanine. It was found that alkaptonuria could be as well produced by tyrosine- as by phenylalanine-rich diets, in preliminary experiments with Drs. Auerbach and LeMay (these studies did not substantiate the development of arthritis in rats during the long continued alkaptonuria (3)). Homogentisate was measured in the urines of 27 rats fed the high tyrosine diet as long as 7 weeks. Animals were killed at intervals during this time for measurement of the levels of the appropriate liver enzymes for correlation with the degree of observed alkaptonuria.

Procedures. Animals. Male albino Slonaker rats weighing 140-250 g were used. The rats were fed, depending on their weights, 10-15 g/day of powdered Purina Rat Chow containing 5% L-tyrosine. Urines were collected over 24-hour periods in flasks containing 2 ml of 50% glacial acetic acid. *Analysis of Urines.* Homogentisic acid was determined by the iodometric titration method of Neuberger(4). *p*-Hydroxyphenylpyruvate (pHPP) was determined by converting it to the enol borate complex(5). To the urine sample was added 3 ml of 1 M boric acid in 2 M arsenate at pH 6.5. The solution was diluted to a final volume of 3.5 ml and after 5 minutes the optical density was read at 310 mμ against a blank which contained every component except boric acid. pHPP ($\epsilon_{310\,m\mu} = 1.24 \times 10^4$) was either absent or present in less than 0.1 mg in several urine samples containing homogentisic acid. Therefore pHPP was not systematically followed in these experiments. *Assay of Enzymes.* All enzyme assays were determined in fresh supernatant fractions of 10% liver homogenates. The livers were homogenized in .14 M KCl in 0.005 N NaOH. The enzyme protein was the sole limiting factor in each of the assay reactions. Activities were expressed as μmoles of substrate reacting/hour/g of dry liver. Tyrosine-α-ketoglutarate transaminase was assayed by a spectrophotometric method which depended upon

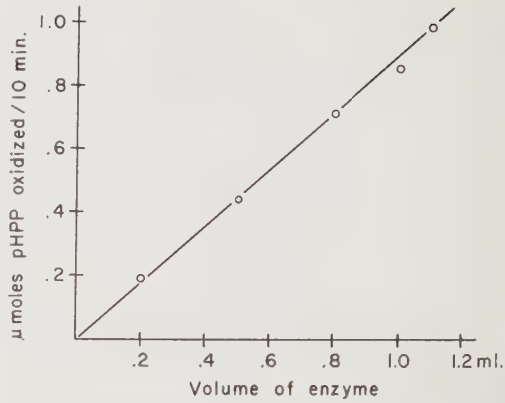

FIG. 1. Activity-concentration curve of rat liver pHPP oxidase measured by the disappearance of the enol-borate complex of pHPP. The enzyme was assayed at 25° ± 1°C in a 1 cm quartz cell containing 0.4 μmole pHPP, an excess of a preparation of kidney keto-enol tautomerase ($k = 1.0$) free of pHPP oxidase(5), 0.57 M borate, pH 8.0 in a final volume of 3.5 ml. The pHPP was allowed to equilibrate for 5 minutes in the borate buffer in the presence of the keto-enol tautomerase before addition of the oxidase. The reaction was initiated by the addition of the rat liver extract. The disappearance of pHPP ($\epsilon_{310} = 9.85 \times 10^3$) was followed for 5 minutes by readings of optical density at 310 mμ, against a blank containing all components of the reaction mixture except pHPP.

the appearance of the enol-borate complex of pHPP(6). *p*-Hydroxyphenylpyruvate oxidase was assayed by spectrophotometric method with slightly different conditions from those described by Hager, Gregerman and Knox(7). The conditions used are given in the caption for Fig. 1, which shows the proportionality of activity to the amount of enzyme used in this assay. Homogentisate oxidase was assayed by the manometric method described by Knox and Edwards(8), with the extra addition of 10 μmoles of glutathione to the reaction mixture to insure full activity. The rate of oxygen uptake was proportional to the amount of enzyme used over a 4-fold range.

Results. The majority of the rats began to excrete homogentisate 3 to 4 days after they were placed on the high tyrosine diet. The mean 24-hour excretions of homogentisate during the period of 7 weeks, constructed from 67 urine collections on 27 animals, are shown in Fig. 2. During the first month the amount of homogentisate excreted rose slowly to a peak of about 100 mg/day and

TABLE I. Correlation of Enzyme Levels in the Liver with Homogentisic Acid Excretion by Male Rats on a High Tyrosine Diet.*

Treatment	Homogentisic acid (mg in 24 hr urine)	Tyrosine transaminase	pHPP oxidase	Homogentisate oxidase
		(Mean ± S.E.)		
Purina chow diet		159 ± 21 (23)†	260 ± 25 (16)	565 ± 22 (15)
High tyrosine diet				
Group I	1 ± .48 (8)	104 ± 25 (8)	233 ± 31 (5)	411 ± 76 (8)
II	27 ± 4.5 (8)	139 ± 45 (8)	282 ± 50 (6)	400 ± 48 (8)
III	81 ± 8.9 (11)	173 ± 40 (11)	268 ± 32 (10)	358 ± 27 (11)

* Enzyme activities are expressed as μmoles of substrate converted/g dry wt of liver/hr. All assays were carried out at 25°C, except homogentisate oxidase which was measured at 37.7°C.
† No. of animals.

then declined. Fewer animals were observed in the late period, so the decline in amount may not be significant.

The rats were grouped according to the amount of homogentisate they individually excreted shortly before they were killed for enzyme assays, regardless of the length of time they were on the diet. Group I included all rats that excreted less than 5 mg of the acid in 24 hours, Group II included rats that excreted from 5-50 mg, and Group III included the rats which excreted more than 50 mg of the acid. The mean levels in control rats and in the 3 groups of experimental rats of homogentisate excreted and of the liver enzyme levels are given in Table I. No significant changes in the levels of *p*-hydroxyphenylpyruvate oxidase occurred. Rats on the experimental diet had lower activities of the homogentisate oxidase and tyrosine transaminase than those on a stock diet. No explanation of the lowered enzyme levels in these animals can be offered, although it might be mentioned that on this diet they grew like the controls and at the end of 8 weeks showed an average gain of 40% of the initial body weight. The homogentisate oxidase levels in the 3 groups on the experimental diet were not significantly different from each other. The tyrosine transaminase levels were lowest in Group I, intermediate in Group II, and highest in Group III, paralleling the amount of homogentisate excreted by these groups. Exactly the same relationship between enzyme activity and alkaptonuria was found when groups of rats sacrificed early and late in the experiment were compared.

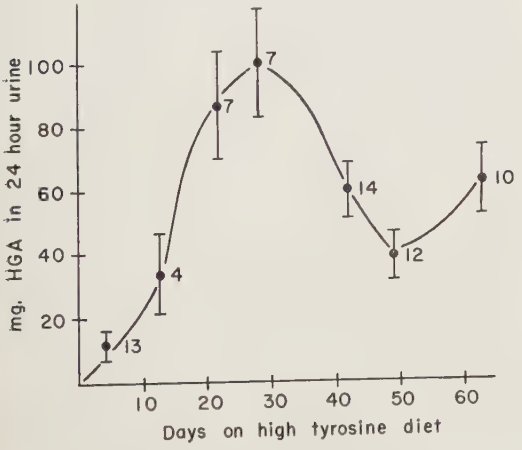

FIG. 2. Excretion of homogentisic acid by rats on high tyrosine diet. The mean homogentisic acid content in 7 groups of urine collections from male rats on a 5% L-tyrosine diet are represented by the dots. The lengths of the bars are equal to 2 × S. E. The number of urine collections at each interval is indicated by the number near each dot.

The ratio of tyrosine transaminase activity (the effective source of homogentisate) to the homogentisate oxidase activity (which disposed of homogentisate) should determine the steady state concentration of homogentisate available for urinary excretion. These ratios, calculated from the enzyme assays on individual animals and then averaged, are given in Table II for the controls and for each of the 3 groups of rats. There are highly significant differences in the ratios for the different groups, with the higher ratios, representing a surplus of homogentisate formation over disposal, being present in the groups excreting more homogentisate.

Discussion. Experimental alkaptonuria provides an excellent opportunity for the study of the relation between an altered living

TABLE II. Correlation of Ratio of Tyrosine Transaminase Activity to Homogentisate Oxidase Activity in the Liver with Homogentisic Acid Excretion.

Rats	Homogentisic acid (mg in 24 hr urine) (Mean ± S.E.)	Tyrosine transaminase / Homogentisate oxidase (Mean ± S.E.)
Controls	——	.24 ± .020 (14)
Group I	1 ± .48 (8)*	.26 ± .022 (8)
II	27 ± 4.5 (8)	.36 ± .045 (8)
III	81 ± 8.9 (11)	.49 ± .036 (11)

Statistical significance of the differences in enzyme levels between groups, as evaluated by the *t* test, is as follows: $P < .1$ for Group I *vs* Group II; $P < .05$ for Group II *vs* Group III; $P < .001$ for Group I *vs* Group III.

* No. of animals.

state and the changed enzyme levels in tissues. All the relevant enzymes occur predominantly in one organ, the liver, and they all can be measured. Any increased formation or decreased degradation of homogentisate producing a greater supply would be immediately reflected in an increased rate of renal excretion, since in man, at least, homogentisate is cleared by the kidney at the glomerular filtration rate(10). The enzymes concerned with both the formation and the degradation of a metabolite must be measured to identify the critical enzyme changes causing the accumulation of the metabolite. The occurrence of physiologically significant adaptive changes in the enzyme levels will be revealed by the appearance of alkaptonuria, and a quantitative comparison can be made between the degree of the enzyme change and the amount of homogentisate excreted. Finally, the absolute values of enzymic rates measured *in vitro* have no direct relation to the rates of the reactions which occur *in vivo,* and the identification of the limiting reactions must depend upon the correlation of enzyme changes with the metabolic changes.

The decrease in homogentisate oxidase without change in the pHPP oxidase which occurred when the rats were given the experimental diet was the sort of metabolic adaptation which might have led to alkaptonuria. That it did not (Group I) suggests that homogentisate oxidase was not the limiting reaction under these conditions. The fact that no pHPP was excreted and that the unchanging activity of pHPP-oxidase could transmit the effects of the increased activity of the transaminase preceding it in the metabolic pathway (Groups II and III) suggests that pHPP oxidase activity was also greater than that of the other enzymes under the conditions present in the cell. Tyrosine transaminase, the effective source of homogentisate in this metabolic sequence, and homogentisate oxidase, the degradative reaction for homogentisate, were present in near rate limiting amounts. This is indicated by the absence of alkaptonuria when the amounts of both these enzymes were reduced by the experimental diet, so long as the ratio of their activities remained the same as in normal animals (Group I), and by the appearance of alkaptonuria when the ratio of transamination to homogentisate degradation increased (Groups II and III).

In all known forms of alkaptonuria there is a predominance of homogentisate formation over its metabolic removal, and renal defects do not cause the condition. In the hereditary form of human alkaptonuria the discrepancy between formation and removal is referrable to decreased removal due to relative inactivity of the genetically controlled homogentisate oxidase. The chemical inhibition of this enzyme by α-α-dipyridyl *in vivo* has produced a similar form of alkaptonuria (9). In the experimental alkaptonuria studied here the predominance of homogentisate formation over its removal is referrable to increased formation due to an adaptive increase in the tyrosine transaminase, possibly the result of the high substrate feeding(6). The amount of this increase (Group I compared with Group III) represented an increase in the potential transaminating ability of a whole rat from 15 mmoles to 24 mmoles per day, equivalent to the formation of 1.5 g of extra homogentisate per day. Less than 10% of this amount would have to escape degradation by homogentisate oxidase for the observed excretion of up to 100 mg of homogentisic acid per day to occur.

Summary. Experimental alkaptonuria of rats maintained on a high tyrosine diet is associated with an increased ratio of activity of

the tyrosine transaminase to activity of the homogentisate oxidase in the liver. This type of alkaptonuria is therefore the result of an ádaptive increase in relative rate of homogentisate formation, in contrast to the relative decrease in rate of homogentisate degradation believed to be responsible for hereditary alkaptonuria in man.

1. Papageorge, E., and Lewis, H. B., *J. Biol. Chem.*, 1938, v123, 211.

2. Knox, W. E., in *Symposium on Amino Acid Metabolism*, edited by W. D. McElroy and B. Glass, Johns Hopkins University Press, Baltimore, 1955, p836.

3. Brand, H. H., *Inaugural-Dissertation*, Friedrich-Wilhelms-Universität zu Berlin, Konrad Triltsch, Würzburg-Aumühle, 1939.

4. Neuberger, A., *Biochem. J.*, 1947, v41, 431.

5. Knox, W. E., and Pitt, B. M., *J. Biol. Chem.*, 1957, v225, 675.

6. Lin, E. C. C., and Knox, W. E., *Biochim. Biophys. Acta*, in press.

7. Hager, S. E., Gregerman, R. I., and Knox, W. E., *J. Biol. Chem.*, 1957, v225, 935.

8. Knox, W. E., and Edwards, S. W., *ibid.*, 1955, v216, 479.

9. Suda, M., Takeda, Y., Sujishi, K., and Tanaka, T., *Med. J. Osaka Univ.*, 1951, v2, 595.

10. Neuberger, A., Rimington, C., and Wilson, J. M. G., *Biochem. J.*, 1947, v41, 438.

11. Knox, W. E., Auerbach, V. H., and Lin, E. C. C., *Physiol. Revs.*, 1956, v36, 164.

Received September 5, 1957. P.S.E.B.M., 1957, v96.

Role of Potassium in Regulation of Coronary Blood Flow.* (23521)

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Katz and Lindner(1) have shown that small increments in the concentration of potassium (K) in defibrinated blood perfusing the fibrillating dog heart result in coronary vasodilation, whereas large increments of K produce vasoconstriction. Dawes(2) observed a similar relationship between blood K concentration and changes in arterial flow in the perfused hind limbs of cats and dogs. Since small increases in K levels decrease vascular resistance and since K is released from actively contracting muscle(3,4), Dawes(2) suggested that the K ion originating in the muscle may be responsible for the increased flow which accompanies muscular contraction. The present study was undertaken to determine to what extent the coronary vasodilation, which occurs during increased metabolic activity of the myocardium, could be accounted for by the release of K from muscle cells.

Methods. Ten dogs weighing 14.6 to 20.7 kg were used in these experiments. The animals were anesthetized with sodium pentobarbital (30 mg/kg) and the chest was opened in the fourth left intercostal space. Lung inflation was maintained by positive pressure artificial respiration and heparin (180 mg) was given to prevent blood clotting. The coronary sinus was cannulated via the right atrium and the venous outflow directed through rubber tubing into the right external jugular vein. The left coronary artery was cannulated with an Eckstein cannula(5) and received blood from the right common carotid artery via a pump perfusion system which maintained a constant perfusion pressure independent of aortic pressure. Mean coronary blood flow (CBF) was measured by an optically recording rotameter and mean perfusion pressure (PP) and phasic aortic pressure (AP) were recorded by modified Gregg manometers. In the first set of experiments several concentrations of KCl were infused proximal to the rotameter (to insure complete mixing of blood and infusate) at a constant rate of 0.93 cc per minute. Concentrations of KCl were used which were calculated to increase the animal's serum K level by 1.5, 3.0 and 6.0 meq/l. Recordings of CBF, AP, and PP were taken 2 min-

* Supported by grant from Natl. Heart Inst., U.S.P.H.S.

TABLE I. Effect of Intracoronary Administration of Potassium on Coronary Blood Flow.

Exp. No.	Infused K^+, meq/min.	Coronary artery K^+, meq/l		Coronary sinus K^+, meq/l		Coronary blood flow, cc/100 g perfused heart/min.		% change CBF*
		Control	Exp.	Control	Exp.	Control	Exp.	
1	.13	2.56	4.23			100.5	114.1	+13.5
4	.13	3.28	4.37	3.29	3.89	72.0	71.8	− .3
6	.13	3.05	4.39	2.87	3.70	54.8	54.6	− .4
8	.13	2.50	5.34			102.3	110.4	+ 7.9
7	.13	3.39	5.40	3.26	4.80	41.6	58.0	+39.4
6	.26		5.80		4.80	49.4	59.9	+21.3
8	.26	3.35	6.20			106.3	116.5	+ 9.6
1	.26		7.48			94.0	100.7	+ 7.1
7	.26		7.50		6.10	62.8	59.9	− 4.6
2	.26	3.72	8.33			59.0	71.1	+20.5
1	.52		8.96			86.7	104.4	+20.4
10	.52	3.60	9.10	3.55	8.70	128.5	152.7	+18.8
6	.52		9.50		9.10	50.1	67.9	+35.5
8	.52	3.00	10.00			101.7	114.3	+12.4
4	.39		10.10		9.67	67.7	74.2	+ 9.6
2	.52		12.10			56.8	71.9	+26.6

* Plus (+) indicates an increase and minus (−) a decrease.

utes after the start of the infusion. At this time flows were stabilized at a new level. Arterial blood samples were drawn from the tubing just proximal to the inflow cannula, and coronary sinus blood samples were taken simultaneously from the tubing distal to the coronary sinus cannula. K concentrations were determined by flame photometry. A second recording of CBF, AP, and PP was taken immediately after the blood samples were collected. Measurements reported here represent the average of the records taken before and after the blood collection. The perfusion with KCl was then stopped and the CBF allowed to return to the pre-perfusion level. Control records of CBF, AP and PP and control samples of coronary arterial and venous blood were taken prior to each infusion of K. Control infusion of 0.9% NaCl at a rate of 0.93 cc/min. was without significant effect on CBF.

In the second set of experiments, CBF was increased by methods which augment myocardial metabolism and by asphyxia. The rate of cardiac metabolism was elevated by 1) intracoronary administration of 2,4-dinitrophenol (1.3-2.7 mg/min.) and epinephrine (9.3-18.6 μg/min.) and 2) constriction of the aorta distal to the left subclavian artery (to increase cardiac work). Asphyxia was induced by temporarily stopping the respirator. Recording of CBF, PP, AP and blood sampling were conducted as described above. After each experiment the perfused portion of the heart was stained, excised and weighed, and CBF has been expressed as flow per 100 g of perfused heart muscle.

Results. In the first group of experiments (Table I) a constant infusion of KCl produced a measurable increase in CBF in 13 of 16 experiments, with an average increase of 17.7%. The rise in flow was not related to the concentration of K added to the perfusate, nor to the final plasma K concentration. No vasoconstriction was observed even when plasma K levels reached 12.1 meq/l. The infusions of KCl produced no significant alteration in heart rate or arterial pressure.

In the second group of experiments, infusion of 2,4-dinitrophenol or epinephrine, production of asphyxia, or increased arterial resistance resulted in moderate to marked increases in CBF (Table II). The average increase in CBF with asphyxia was 60.5%, with epinephrine 63.6%, and with DNP 35.4%. The results with acute hypertension were variable: a 14% increase occurred in one animal and no significant change occurred in two other dogs. Although flow was significantly increased by several different procedures, no significant changes in coronary sinus plasma K concentrations or in K arteriovenous differences were observed.

Discussion. Intracoronary administration

TABLE II. Effect of Increasing Coronary Blood Flow on Coronary Sinus Blood Potassium Levels.

Exp. No.	Method of increasing CBF	Coronary artery K^+ conc., meq/l		Coronary sinus K^+ conc., meq/l		C.B.F., cc/100 g perfused heart/min.		% change CBF	Change K^+ A-V differences, meq/l
		Control	Exp.	Control	Exp.	Control	Exp.		
4	Asphyxia	3.66	3.25	4.11	3.87	67.2	130.2	+ 93.8	+.17
3	"	3.33	3.46	3.26	3.13	97.4	112.9	+ 15.9	+.26
6	"	3.50	3.48	4.10	4.00	92.2	125.4	+ 36.0	+.08
11	"	2.57	4.47	2.48	3.73	84.5	116.0	+ 96.4	+.83
11	Acute hypertension	2.45	2.58	2.46	2.61	80.5	77.9	− 3.2	+.02
11	*Idem*	2.81	2.89	2.69	2.81	74.0	75.5	+ 2.0	−.04
4	"	3.79	3.61	3.83	3.79	60.1	72.4	+ 20.4	+.14
	Dinitrophenol								
11	(1.3 mg/min.)	2.56	2.46	2.66	2.58	79.7	100.6	+ 26.2	−.12
11	(2.7 ")	3.00	2.52	2.78	2.61	67.2	108.5	+ 61.5	−.31
7	(1.5 ")	3.09	3.09	2.91	3.28	63.5	67.2	+ 5.8	+.35
7	(3.7 ")		3.10		3.16	63.5	94.5	+ 48.8	
10	(2.7 ")	3.55	3.20	3.50	3.15	137.5	185.4	+ 34.8	.00
	Epinephrine								
9	(9.3 μg/min.)	2.60	2.50	2.60	2.60	68.8	110.6	+ 60.6	−.10
11	(18.6 ")	2.84	2.69	2.73	2.20	63.2	130.1	+104.4	+.38
11	(9.3 ")	2.62	2.79	2.50	2.21	79.0	115.3	+ 45.9	+.46
10	(18.6 ")	2.50	2.85	3.50	2.45	109.5	156.5	+ 43.4	+.14

Plus (+) indicates an increase and minus (−) a decrease.

of KCl at rates which elevated plasma K from 1 to 8 meq/l produced small to moderate increases in CBF. Although these results confirm those of Katz and Lindner(1) and of Dawes(2), it is difficult to make quantitative comparisons of their data with ours because they did not measure plasma K concentrations and made almost all of their observations on blood flow following single rapid injections of KCl. With high concentrations of K these investigators(1,2) observed vasoconstriction. The concentration of K that must be reached in the plasma to induce vasoconstriction cannot be accurately determined from their data, but it must be in excess of 12 meq/l, since we observed only vasodilation at this, or lower, concentrations.

Although the heart beating *in situ* is more physiological than a fibrillating heart preparation, flow may conceivably be altered by changes in extravascular compression which would accompany changes in heart rate or force of contraction. However, it is unlikely that extravascular compression was significantly altered in the present study since heart rate and aortic pressure were not affected by the KCl infusion.

Procedures which increase CBF by increasing the oxygen requirements of the heart or by decreasing the oxygen supply to the myocardium were not associated with K release from the myocardium. In fact, with one exception (Exp. 11—asphyxia), negligible changes in K A-V differences occurred and most of these were in a positive rather than negative direction. In the few instances in which the potassium A-V difference decreased, the change was due to a fall in arterial rather than a rise in coronary sinus K concentration. It is possible that the present methods were not sensitive enough to detect the release of K from the heart. If this is true then the concentration of K reached in the vicinity of the arterioles is too small to produce changes in arterial resistance since concentrations as high as 12 meq/l failed to produce changes in blood flow of the magnitude seen with increases in myocardial metabolism or asphyxia.

These observations do not support the hypothesis of Dawes(2) that K release from muscle is responsible for the reduction in vascular resistance associated with increased muscular activity. The nature of the metabolite(s) which operate to adjust blood flow to metabolic requirements remains obscure.

Summary. Intracoronary infusion of KCl sufficient to elevate coronary arterial plasma K concentration to levels of from 4.23 to 12.10 meq/l produced increases in CBF averaging

17.7%. The changes in CBF produced by KCl infusion did not parallel the changes in plasma K concentration. Infusion of 2,4-dinitrophenol or epinephrine, asphyxia, or increased aortic pressure (all factors which are known to increase myocardial oxygen consumption and coronary blood flow) did not result in the release of K from the myocardium. Therefore, it appears unlikely that K release from active myocardium is responsible for adjustment in coronary resistance which accompanies changes in metabolic activity of the myocardium.

1. Katz, L. N., and Lindner, E., *Am. J. Physiol.*, 1938, v124, 155.

2. Dawes, G. S., *J. Physiol.*, 1941, v99, 224.

3. Fenn, W. O., Cobb, D. M., Manery, J. F., and Bloor, W. R., *Am. J. Physiol.*, 1938, v121, 594.

4. Fenn, W. O., *ibid.*, 1939, v127, 356.

5. Eckstein, R. W., McEachen, J. A., Demming, J., and Newberry, W. B., *Science*, 1951, v113, 385.

Received September 9, 1957. P.S.E.B.M., 1957, v96.

Reactivity of Sulfhydryl and Disulfide Groups in Serum of Man.* (23522)

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Sulfhydryl-containing compounds such as cysteine and cysteinamine have been shown to be very effective in protecting against the lethal effects of ionizing radiations(1-3). As one approach to understanding the mechanism underlying this protection we have measured in human serum the levels of sulfhydryl (–SH) and disulfide (–S–S–) and their reaction to ultraviolet (UV) light, detergent, and electrolysis.

Materials and methods. The serum used in these studies was freshly prepared from the blood of young (20-25 years) and older (40-50 years) human subjects and from various types (metastatic carcinoma of the throat and rectum) of cancer patients from the Ghent University Clinic. The sulfhydryl and disulfide determinations were accomplished using one of the amperometric methods described by Kolthoff *et al.*(4). The –SH determination was carried out in an ammonia buffer (0.1 M NH_4OH, 0.3 M NH_4NO_3); after deaeration of the buffer solution with purified nitrogen(5) the serum was added and titrated with a 0.002 or 0.001 M solution of $AgNO_3$ at –0.30 volt, *vs.* a standard calomel electrode. The total volume was 20 ml. The –S–S– determination was carried out in ammoniacal medium in the presence of sulfite (0.1 M NH_4OH, 0.1 M NH_4NO_3, 0.2 M Na_2SO_3). The $AgNO_3$ solution and the potential were identical to that used in the –SH determination. The nitrogen content of the serum was determined by an amperometric method which makes it possible to analyze very minute quantities of serum (down to 0.02 ml) in 15 minutes(6). UV irradiation. A UV lamp (type 16200, Hanovia Chemical and Manufacturing Co., Newark, New Jersey) was used to irradiate a quartz cell with a diameter of 1.27 cm and a length of 8.40 cm. The distance from the cell to the lamp was 6 cm. An aliquot of serum, diluted 1:16 with air-free phosphate buffer (0.1 M Na_2HPO_4, 0.01 M NaH_2PO_4; pH 7.29), was pipetted into the cell, which was closed hermetically after purified nitrogen had been passed over the sample(4). A current of cold air was blown over the cell in order to avoid a rise of temperature during the experiment; after 2 hours of irradiation the temperature rose 1°C. Aluminum foil was used as a reflector behind the cell. Anionic Detergent. To test the effect of an anionic detergent upon –SH and –S–S– groups, 0.5 ml of serum was placed in 20 ml

* This work performed under the auspices of the U. S. Atomic Energy Com. under the Belgian-American Agreement Concerning Atomic Energy.

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of 7.5 x 10^{-5} M sodium laurylsulfate for 15 minutes. The –SH and –S–S– determinations were carried out according to the technic mentioned above. Electrolysis. Different methods have been used in order to reduce –S–S– in proteins. One of these is reduction with sodium amalgam (10%) in acid or basic medium. Using this method we found that after 30 minutes the –SH value of serum rose from the normal 6.7 mg/100 ml only to 8.7 mg/100 ml, which was not adequate for our purposes. Hata(7) has described a technic for oxidizing cysteine to cystine in different acid media by electrolysis. By applying a modification of this technic to serum proteins we were able to obtain a high degree of reduction of –S–S–. Serum was used in a 1:20 dilution in a medium containing 0.05 M HCl and 0.5 M KCl. The applied potential was 90 volts, over an agar bridge saturated with KCl; the current was 40 ma. The reaction was carried out in an air-free medium(4), and the thermostat was set at 25.00° C ± 0.02.

Results. After 2 hours of UV irradiation the –SH content of a sample of normal serum was 42 mg/100 ml. When the proteins of the serum were precipitated with 20% sulfosalicylic acid, the filtrate contained only 1.3 mg –SH, which indicates that the –SH of the serum is still contained in proteins of high molecular weight.

To investigate the behavior of –S–S– groups under influence of UV light, 4 ml of 5 x 10^{-5} M cystine solution in 0.05 M HCl was placed in a quartz cell and diluted to 8 ml with air-free phosphate buffer. After irradiation of the solution for 30 minutes, 0.724 x 10^{-5} moles –S–S– was reduced to –SH, 2.07 x 10^{-5} moles of –S–S– was unchanged, and 44% of –S–S– was lost by oxidation or breakdown of the molecule. After UV irradiation of a 7% albumin solution diluted 1:16 with the air-free phosphate buffer, the content of –SH increases with the duration of irradiation (Fig. 1). In the case of normal serum a greater amount of –SH and –S–S– is found after irradiation. We shall refer to this increase in detectable –SH and –S–S– as reactivity. Fig. 2 gives an example of normal serum titrated after different periods of irradiation. During these experiments it was also found that non-irradiated serum contains reactive –S–S– groups, which are amperometrically detectable. When one-day-old serum was used, the –SH and –S–S– contents were lower than that of fresh serum, and the reactivity of the groups was lower.

In cases of cancerous serum the same phenomenon was manifested. However, the reactivity is much greater; there are more titratable –SH and –S–S– groups after the irradiation period, compared with normal serum, and fewer –SH and –S–S– groups detectable in the untreated serum (Fig. 3).

In young persons up to 25 years the average –SH content of the serum is 6.8 mg/100 ml serum (mean of 200 individuals, range 5.6 to 8.6 mg/100 ml serum). In older persons (40 years of age) the average content is 5 mg/100 ml serum (mean of 200 individuals, range 4.3 to 6.9 mg/100 ml serum). The reactivity of the –SH groups after UV radiation is, however, much greater in older persons than in the case of younger ones.

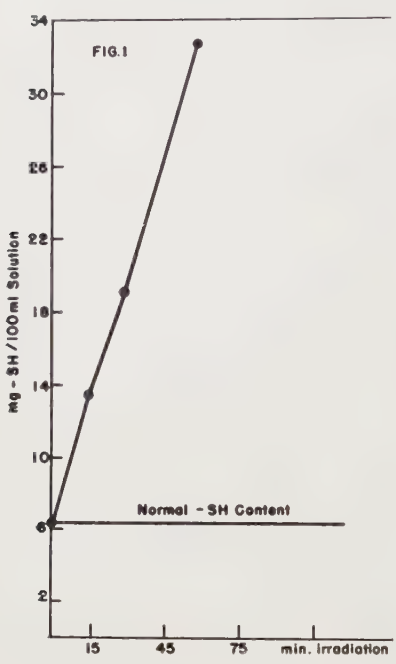
The average –SH content of cancerous serum is 3.3 mg –SH/100 ml serum (mean of 150 individuals, range 2.3 to 5.4 mg/100 ml serum), the reactivity of the groups is much higher compared with the sera of normal persons. The –S–S– content averages 7.2 mg/100 ml serum (range 4.0 to 9.2 mg/100 ml serum) for normal persons, and 12.1 mg/100 ml serum (range 7.3 to 14.0 mg/100 ml serum) in the cases of cancerous serum. The overlapping of the –SH and –S–S– contents between normal and cancerous sera makes a differentiation on that basis difficult; the error factor is 12%.

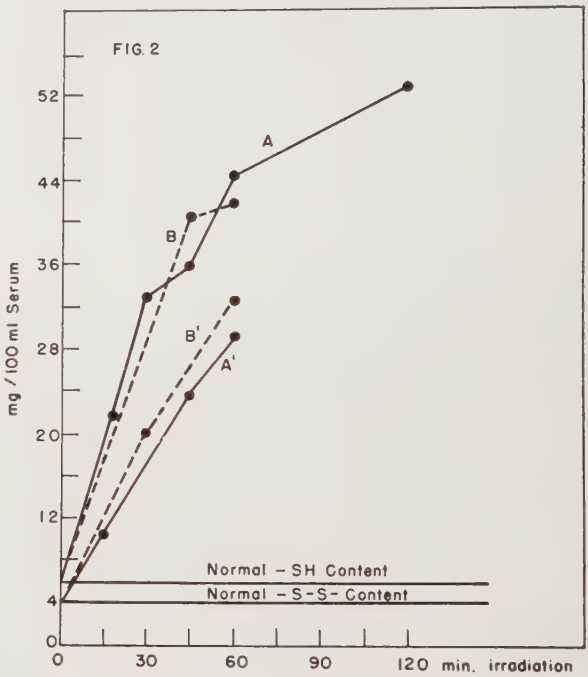
The greater amount of –SH and –S–S– found after irradiation can be explained by the unfolding property of the molecules, by which more of these groups become free, a reaction that occurs also when the molecules are under influence of a detergent.

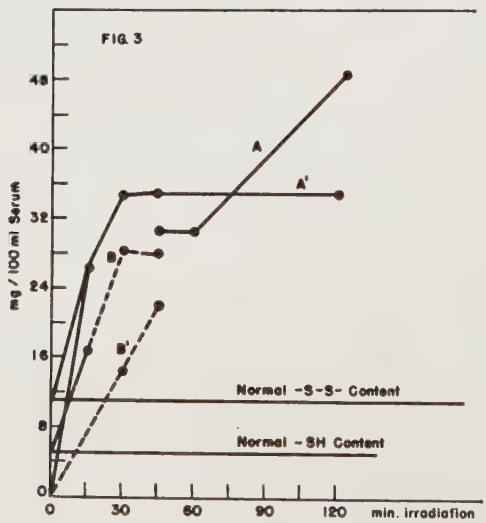
Cystine and serum behave differently under the influence of UV light; cystine is destroyed, while serum gives more titratable –SH and –S–S– groups.

The reactivity of these two groups is of great significance if correlated with the work of Bacq *et al.* (1) and Maisin *et al.*(2) who studied the protective properties of sulfhy-

dryl compounds such as cysteinamine (β-mercaptoethylamine, Bécaptan). The effectiveness of this compound can perhaps be explained by absorption of radiation energy, as shown previously in the case of cysteine. Another, and more likely possibility(8) is that complexes are formed between the compound and the other –SH and –S–S– groups in the







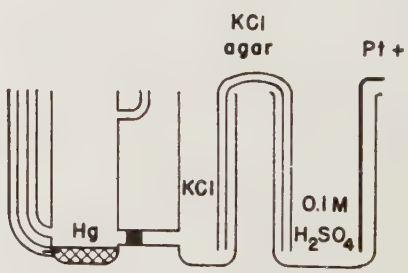


FIG. 4 ■ Sintered glass plate electrolysis cell

FIG. 1. Amperometric –SH titration of a 7% albumin solution after UV irradiation.

FIG. 2. Amperometric –SH and –S–S– determination of normal serum after UV irradiation; dilution 1 : 16. A, normal –SH content of fresh serum; A′, normal –S–S– content of fresh serum; B, normal –SH content of serum one day old; B′. normal –S–S– content of serum one day old.

Fig. 3. Amperometric –SH and –S–S– determination of cancerous serum after UV irradiation; dilution 1 : 16. A, normal –SH content of fresh serum; A′, normal –S–S– content of fresh serum; B, normal –SH content of serum one day old; B′, normal –S–S– content of serum one day old.

FIG. 4. No text.

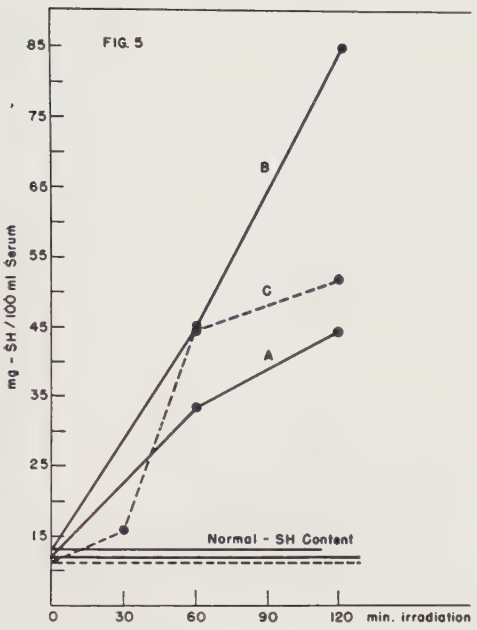


FIG. 5. Amperometric –SH titration of normal serum (A and C) and a 7% albumin solution (B) after electrolysis; 1 ml in 0.05 M HCl and 0.5 M KCl. Total volume 20 ml, current 40 ma, 90 volts.

serum. This complex formation can be shown polarographically; the stoichiometric equivalent could not be determined. In this reaction the amino groups seem to play an important role.

Under the influence of a detergent, sodium laurylsulfate, an effect similar to that of UV light was noticed. However, the amounts of –SH and –S–S– released were lower, and the difference between sera of young and older persons was also less distinct.

If the serum proteins were precipitated in pH 3.7 acetate buffer (0.1 N acetic acid, 0.1 N sodium acetate) and redissolved in pH 9 ammonia buffer, the –SH and –S–S– values were higher than those obtained by direct determinations under the influence of the detergent without any precipitation. These experiments make it clear that under the influence of a detergent the spiral structure of the proteins unfolds, and a greater number of –SH and –S–S– groups can be detected.

After 4 hours of electrolysis (Fig. 4) 92 mg of –SH per 100 ml of a normal serum were detected (Fig. 5). The reaction of a 7% albumin solution is given for comparison. The results of these experiments show again that sera of younger persons have a lower reactivity compared to those of older persons. However, as in the experiments with sodium laurylsulfate, the difference is less marked than in the experiments with UV.

After electrolysis in basic medium (0.5 M NH_4OH and 0.25 M NH_4Cl), no greater amounts of –SH and –S–S– could be detected; *e.g.*, after 2 hours no –SH could be determined and a cathodic current appeared. The nature of this reaction has not been determined.

Summary. 1) Reactivity of sulfhydryl and disulfide groups in normal and cancerous sera was studied under the influence of UV radiation, detergents, and electrolysis. We were able to demonstrate that serum as such contains reactive –S–S– groups, and that the reactivity of –SH and –S–S– differs according to age and for normal and cancerous serum. 2) The greater amount of –SH and –S–S– found after irradiation could be due to unfolding of the spiral structure of the protein and consequent release of the residual bonds. The energy required for normal serum is greater in the case of younger people (up to 25 years) than for older people (aging effect) and also greater for normal serum than for cancerous ones. 3) Sodium laurylsulfate also greatly increased the –SH and –S–S– content, which can be explained by the unfolding property of the molecule. The electrolysis of serum in acid medium made it possible to reduce –S–S– groups in proteins; the response of serum to this treatment is described. The possible relation of these results to the protective action of –SH against irradiation is discussed.

1. Bacq, Z. M., Dechamps, G., Fisher, P., Herve, A., Le Bihan, H., Lecomte, G., Pirotte, M., and Rayet, P., *Science,* 1953, v117, 633.

2. Maisin, G., Mandart, M., Lambert, S., and Maisin, H., *Compt. rend. Soc. Biol.,* 1953, v148, 362.

3. Patt, H. M., Tyree, E. B., Straube, R. L., and Smith, D. E., *Science,* 1949, v110, 213.

4. Kolthoff, I. M., Stricks, W., Morren, L., and Heyndrickx, A., *J. Nat. Cancer Inst.,* 1954, v14, 1233.

5. Meites, L., Meites, T., *Anal. Chem.,* 1948, v20, 984.

6. Kolthoff, I. M., Stricks, W., Morren, L., *Analyst*, 1953, v78, 405.

7. Hata, T., *Bull. Research Inst. Food. Sci.*, Kyoto Univ., 1950, v3, 63.

8. Eldjarn, L., and Pihl, A., *Progress in Radiology*, pp. 249-259; Mitchell, J. S., Holmes, B. E., and Smith, C. L., eds. Oliver and Boyd, London, 1956.

Received September 9, 1957. P.S.E.B.M., 1957, v96.

Humoral Conditioning for Production of Acute, Massive Myocardial Necroses by Neuromuscular Exertion.* (23523)

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Previous experiments have shown that 2-methyl-9(α)-chlorocortisol (Me-Cl-COL), administered in combination with either basic or acid phosphates, results in acute, massive myocardial necroses in the rat(1). The possible relationships between this experimental lesion and the cardiac infarcts of man have not yet been studied. Since it is well known that neuromuscular effort is likely to precipitate myocardial infarction in predisposed people, the question arose whether this would also be the case in rats sensitized by threshold amounts of Me-Cl-COL and phosphate.

Materials and methods. Eighty female Sprague-Dawley rats, with an average initial body-weight of 101 g (range: 95-110 g), were subdivided into 4 equal groups, as indicated in Table I. Me-Cl-COL† was administered subcutaneously, in the form of its acetate, as a microcrystal suspension of 50 μg in 0.2 ml of water, once daily. NaH_2PO_4 was given by stomach tube, as a 15% aqueous solution, at the dose of 2 ml, twice daily. Neuromuscular strain was induced by maintaining the rats on a board with adhesive tape, for a period of 17 hours, starting 78 hours after the initiation of the Me-Cl-COL and NaH_2PO_4 treatment. The animals of all 4 groups were killed with chloroform, 5 hours after the release of the restrained rats. The hearts and the kidneys of half of the animals in each group were fixed in Susa solution for subsequent staining with hematoxylin-eosin, and those of the other half in neutral formalin for the subsequent demonstration of calcium deposits by v Kossa's silver nitrate stain. For both of these purposes the tissues were embedded in paraffin.

Results. The principal results of these experiments are summarized in Table I.

Upon naked-eye inspection at autopsy, the most striking change was the presence of large, light-yellow patches throughout the myocardium in all the animals of Group IV (Fig. 1). Similar, though less extensive lesions were also seen in 20% of the rats in Groups II and III, but not in Group I.

TABLE I. Humoral Conditioning for Production of Acute, Massive Myocardial Necroses by Neuromuscular Exertion.

Group	Treatment	Myocardial lesions Incidence	Myocardial lesions Severity*	Nephrocalcinosis Incidence	Nephrocalcinosis Severity*	Mortality (%)
I	Me-Cl-COL	0	0	0	0	0
II	" + Restraint	20	.3	0	0	0
III	" + NaH_2PO_4	20	.4	100	2.0	0
IV	" + Restraint + NaH_2PO_4	100	2.6	100	2.0	50

* Severity of the lesions was assessed both macro- and microscopically, in terms of an arbitrary scale of 0-3; figures in this column are the means of these readings.

* Supported in part by a grant from the Gustavus and Louise Pfeiffer Research Foundation and by a Grant from the Muscular Dystrophy Association of Canada.

† Generously supplied by The Upjohn Co.

Histologically, the yellowish patches proved to consist of necrotic cardiac muscle, partly infiltrated by polymorphonuclear leukocytes and monocytes (Fig. 2). The histogenesis of

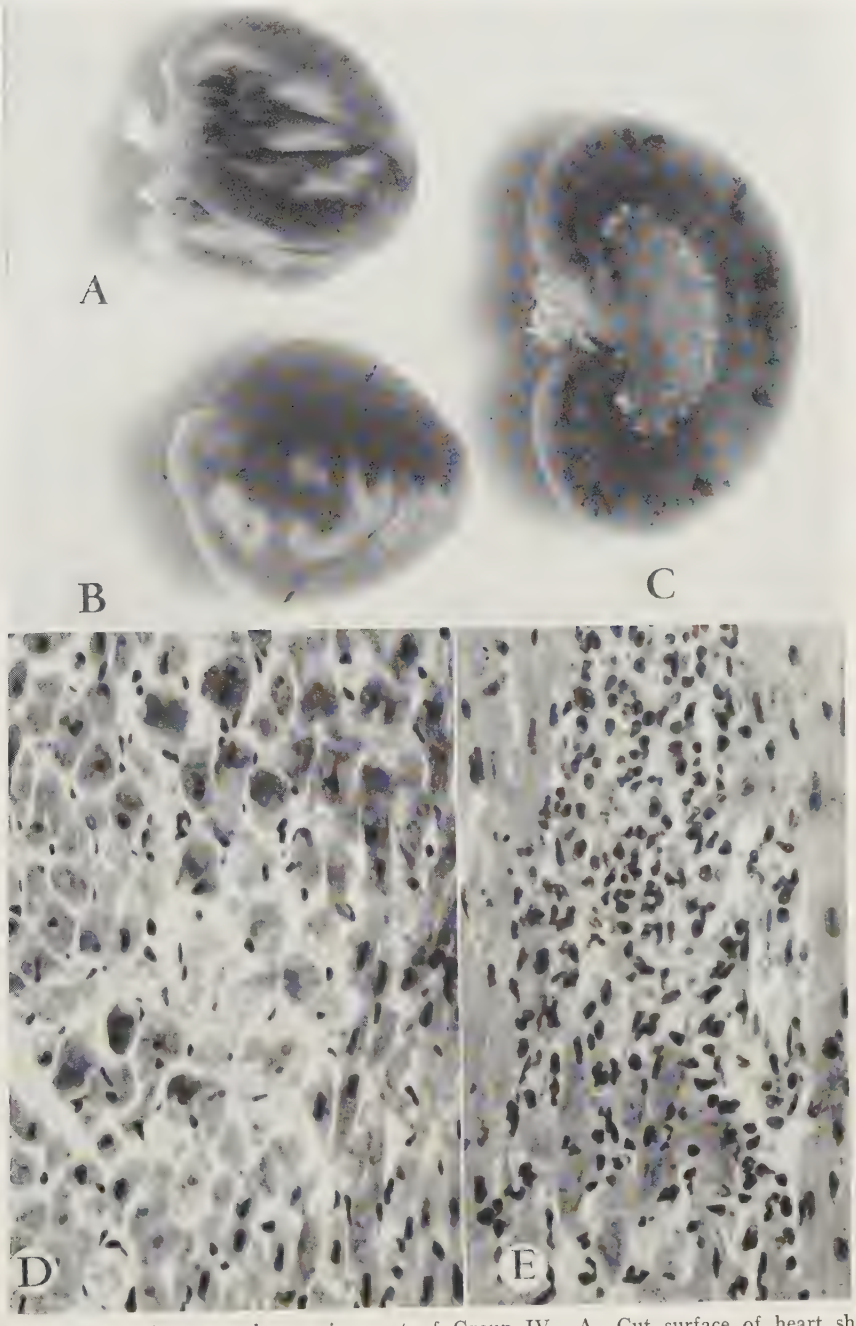

FIG. 1. Characteristic organ changes in a rat of Group IV. A. Cut surface of heart showing multiple large myocardial necroses in wall of ventricle and in papillary muscles as they appear to the naked eye. B. Similar infarcts underneath external surface of the same heart. C. Cut surface of kidney showing white spots of nephrocalcinosis at the cortico-medullary junction. D. Small focus of myocardial necrosis. Some of the affected muscle fibers stain particularly dark with eosin, others are pale and in the process of dissolution (× 320). E. A similar myocardial focus infiltrated with polymorphonuclear leukocytes and monocytes (× 320).

these lesions will be discussed in detail elsewhere; suffice it to mention here that, although histochemically demonstrable, fine granules of calcium were visible in certain areas of the myocardium, there appeared to be no close relationship between these and the necrotic areas. Indeed, most of the focal necroses contained no histochemically demonstrable calcium.

Nephrocalcinosis occurs readily in rats treated with mineralocorticoids and phosphates(2). Accordingly, this change was pronounced in Groups III and IV of the present series, but absent in Groups I and II, in which Me-Cl-COL was given without excess phosphate. There appeared to be no obvious relationship, however, between intensity of the nephrocalcinosis and the myocardial necroses; the latter were evident in 20% of the rats, both in Groups II and III, although in the former there was no nephrocalcinosis, while in the latter pronounced renal calcification was detectable in every animal.

Deaths occurred only in the animals in Group IV and were preceded by signs of cardiac failure, particularly dyspnea and pronounced cyanosis.

Discussion. Our earlier investigations have shown that even intense nephrocalcinosis, if produced by excess phosphate alone, does not cause myocardial necrosis(1). A lack of relationship between intensity of renal calcification and the cardiac changes is also apparent in the observations reported here. It is clear, furthermore, that Me-Cl-COL alone does not produce either myocardial or renal lesions, but merely "conditions" the organism for the production of such changes by an excess of NaH_2PO_4. It is also evident that, in rats maximally sensitized for the production of myocardial necrosis by conjoint treatment with Me-Cl-COL plus NaH_2PO_4 (and, to a lesser extent, in those sensitized only by Me-Cl-COL), the neuromuscular exertion consequent to forced restraint elicits acute, massive myocardial necroses. It remains to be seen whether the muscular effort or the frustration of forced restraint is most important in the production of these infarct-like changes; in any event, presumably, increased cardiac work during effort is a decisive factor. It is dubious whether the myocardial lesions are true infarcts; they are so acute that it is impossible to determine by histological means whether the death of muscle fibers is due to a primary metabolic derangement in the myocardium, or the secondary result of insufficient blood-supply; indeed, both factors may play a part.

Summary. Following pretreatment with 2-methyl-9(α)-chlorocortisol (Me-Cl-COL), a brief period of neuromuscular effort (induced by force restraint) can elicit acute, massive myocardial necroses in the rat. When NaH_2PO_4 was administered in addition to Me-Cl-COL during the pretreatment period, such changes occurred in 100% of the experimental animals, during or immediately after a period of forced restraint.

Addendum: Since this manscript went to press, it has been possible to show that combined treatment with cortisol and NaH_2PO_4 produces similar massive myocardial necroses in the rhesus monkey. These findings will be published in detail elsewhere; they are mentioned here only because they demonstrate that the principal natural glucocorticoid is also effective and that the primate responds like the rat in this respect.

1. Selye, H., and Renaud, S., *Am. J. Cardiol.,* 1957.
2. Selye, H., and Bois, P., *Am. J. Physiol.,* 1956, v187, 41.

Received September 10, 1957. P.S.E.B.M., 1957, v96.

A Culture Strain (LAC) of Human Epithelial-Like Cells from an Adenocarcinoma of the Lung. (23524)

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A number of tissue culture cell lines derived from human tissues have been reported (1,4). Only two of these cell lines have been isolated from patients with carcinoma of the lung and in neither case was the isolation made directly from the primary tumor. One, the "Maben" cell, was derived from pleural fluid of a patient with an adenocarcinoma of the lung(3), and the other, the Detroit 6 cell, originated from the bone marrow of a patient with lung cancer(5). The purpose of this report is to describe the isolation, maintenance, morphological characteristics and some of the viral susceptibilities of a cell line (strain LAC) derived from a human adenocarcinoma of the lung.

History of cell strain. A right pneumonectomy was performed on a 55-year-old white man with an adenocarcinoma of upper lobe of the right lung on Oct. 19, 1955. Histologically the tumor was composed of large areas of undifferentiated carcinoma (Fig. 1) as well as areas of well differentiated adenocarcinoma (Fig. 2). Mucin secretion by tumor cells was demonstrable with Mucicarmine, the Rhinehart stain, and the periodic-acid Schiff reaction after diastase digestion.

Some of the tumor was treated with trypsin solution according to the method of Youngner(6). Another portion was finely minced and suspended in Eagle's basal medium without trypsin digestion. Both cell preparations were then centrifuged and resuspended in a medium consisting of 40% human serum and 60% Eagle's basal medium. Aliquots were placed in 2-oz. prescription bottles.

Results. Within 2 days, groups of cells were evident on the glass surfaces in all cultures. The cells were elongated and pleomorphic and were fibroblast-like in appearance. They had elongated nuclei with prominent nucleoli (Fig. 3). Scattered through the cultures were groups of larger irregularly shaped cells with nuclei containing coarse chromatin granules and large nucleoli which frequently had a peri-nucleolar halo (Fig. 4). Cell growth was not rapid and only 6 subcultures were made over an 11-week period. Small colonies of epithelial-like cells were first noted in the 6th subculture. These cells rapidly outgrew the slowly multiplying fibroblast-like cells. In Fig. 5, a culture of these epithelial-like cells is seen with a large atypical mitotic figure in center of field. Nuclear pleomorphism and hyperchromatism are evident with several small irregularly shaped nucleoli present. Phagocytosis of degenerating cells by other cells may be seen.

Four cultures of the epithelial-like cells were preserved by freezing so that they would be available if the cell line were lost. Preservation of cell cultures was done using a modification of established technics(7-9). Glycerol, USP, (sterilized by autoclaving at 10 lb pressure for 15 minutes) was added to make a 15% final concentration of glycerol. The stopper was secured to the 2-oz. culture bottle with masking tape and the cells under the glycerol-containing medium were allowed to freeze slowly in a solid carbon dioxide storage chest at approximately –70°C. For use, the culture was rapidly thawed in a 37°C water bath with resulting loss of attachment of the cells to the glass surface. The cells were then sedimented at 500 rpm for 10 minutes in the International, size 2, centrifuge, the supernatant decanted, the cells resuspended in fresh growth medium in a 2-oz. culture bottle and incubated at 36°C. Cell multiplication was evident within 24 hours. It was estimated that ¼ to ½ of the cells survived such prolonged storage.* Shortly thereafter all active cultures of the LAC cell were lost due to a combination of poor cul-

* Other tissue culture cell lines have been preserved in this manner (HeLa (Gey), D189 (Leighton), J96 (Osgood), monkey kidney) for as long as 18 months.

tural conditions and bacterial contamination.

A culture which had been stored frozen for 3 months was thawed and cultivation was resumed. Subsequently the cell line has maintained continuous rapid proliferation for over 14 months. 0.25% trypsin solution was used to suspend cells for passage. The human serum content of the growth medium has been

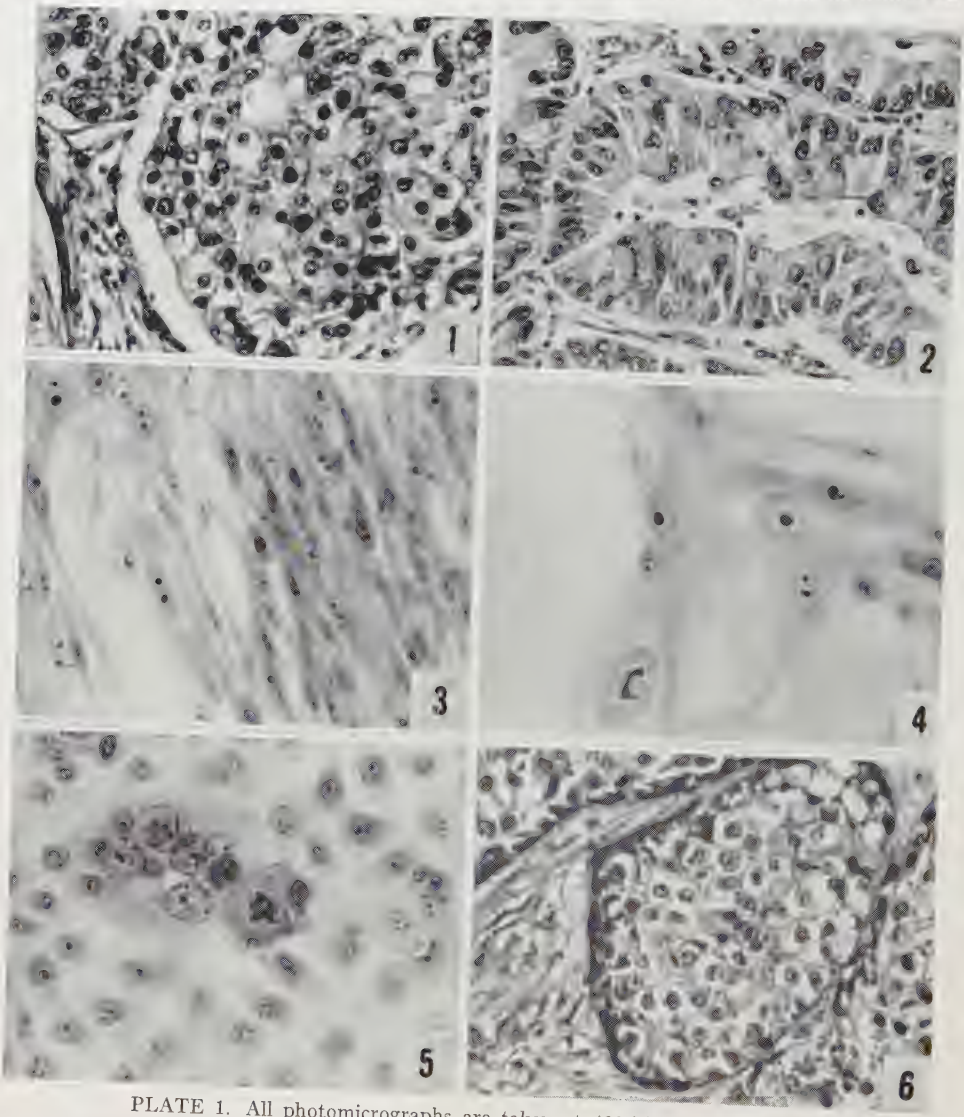

PLATE 1. All photomicrographs are taken at 420 × magnification.

FIG. 1. Section of primary carcinoma of lung in an undifferentiated area. (Hematoxylin & Eosin).

FIG. 2. Section of primary carcinoma of lung with mucin-secreting columnar cells resembling bronchial type epithelium. (H & E).

FIG. 3. Fibroblast-like cells in early tissue culture passages of carcinoma of lung. (Papanicolau stain).

FIG. 4. Large irregularly shaped cells in early tissue culture passages of carcinoma of lung. (Papanicolau stain).

FIG. 5. Epithelial-like cells in later tissue culture passages of carcinoma of lung. Note large atypical mitotic figure near center of field. (Papanicolau stain).

FIG. 6. Tumor in subcutaneous tissue of cortisone-treated irradiated rat after injection of LAC cells. Note resemblance to primary tumor in upper left. (H & E).

TABLE I. Virus Susceptibility of LAC Cell.

Virus	Maintenance medium*	Passages	Cytopathogenesis§	Remarks
Poliovirus—Type I	BME† + 10% ES‡	5	+	
Coxsackie B2	*Idem*	5	+	
" A9	"	1	0	
Vaccinia	"	5	+	
Herpes simplex	"	3	+	Isolated directly from human "cold sore"
Sendai	"	5	0	Cytopathogenesis on 1st passage only
Yellow fever	BME + 4% ES		0	

* Before maintenance medium was added, tubes were rinsed 3 times with serum-free BME.
† Basal Medium Eagle.
‡ Horse serum.
§ Titrations of infectivity were not performed.

decreased to 10% with undiminished rapidity of growth.

Transplantation to rats. It has been possible to transplant the cell line to cortisone-treated irradiated rats both subcutaneously and intraperitoneally with production of tumor nodules(11,12). In the sections of these nodules in the rat, collections of epithelial cells with morphologic features of an undifferentiated carcinoma are seen (Fig. 6). These are similar to the undifferentiated areas in the primary tumor (Fig. 1). In this regard it is of interest that intracellular mucin has been found in these tumor nodules in the rat, and the mucin has the same staining reactions as mucin found in the well-differentiated portions of the original tumor from which the cell line has been derived. It should be noted that tumor nodules with a similar histologic appearance and with intracellular mucin-like material have been produced in rats after injection of HeLa cells.

Susceptibility to viruses. Cultivation of a number of viruses on the LAC cells was attempted. The viruses used included Mahoney strain of Type I poliovirus, Coxsackie A9 and B2†, monkey kidney adapted vaccinia virus, Sendai virus, and the yellow fever virus (17D vaccine strain). Since this part of the study is continuing, a preliminary summary is presented in Table I.

† Coxsackie A9 and B2 viruses were obtained from Dr. Alexis I. Shelokov, monkey kidney adapted vaccinia virus was obtained from Dr. Joel Warren, and Sendai virus was obtained from Dr. Kenneth K. Takemoto.

Discussion. The histogenetic concepts of the derivation of epithelial-like cell strains and transformations of fibroblast-like cells to epithelial-like cells in tissue culture have been discussed in considerable detail recently(1,10, 13) and will not be considered in this report.

A new tissue culture cell line is in some ways analogous to a new bacteriologic medium used for the study of previously uncultured microorganisms. Such cell strains might support and indicate the growth of as yet undetected viruses. The use of a tissue culture cell derived from bronchial type epithelium would seem to be of special value in attempts to isolate agents from respiratory diseases. The viral spectrum of the LAC cell (Table I) is as yet not sufficiently complete to allow final evaluation of the usefulness of the cell; however, further viral studies are now in progress.

Summary. 1) Isolation, maintenance, and morphological features of a cell strain (LAC) derived from a human adenocarcinoma of the lung are described. 2) Preliminary viral studies indicate that the LAC cell is susceptible to poliovirus, Coxsackie B2 virus, vaccinia virus and herpes simplex virus.

The authors would like to acknowledge the technical assistance of Mr. George D. Gardner and Mr. Richard J. Low.

1. Berman, L., and Stulberg, C. S., PROC. SOC. EXP. BIOL. AND MED., 1956, v92, 730.
2. Eagle, H., *ibid.*, 1955, v89, 362.
3. Frisch, A. W., Jentoft, V., Barger, R., and Losli, E. J., *Am. J. Clin. Path.*, 1955, v25, 1107.
4. Jordan, W. S., PROC. SOC. EXP. BIOL. AND MED.,

1956, v92, 867.

5. Berman, L., Stulberg, C. S., and Ruddle, F. H., *Blood,* 1955, v10, 896.

6. Youngner, J. S., PROC. SOC. EXP. BIOL. AND MED., 1954, v85, 202.

7. Scherer, W. F., and Hoogasian, A. C., *ibid.,* 1954, v87, 480.

8. Merryman, H. J., *Science,* 1956, v124, 515.

9. Polge, C., Smith, A. U., and Parkes, A. S., *Nature,* 1949, v164, 666.

10. Leighton, J., Kline, I., and Orr, H. C., *Science,* 1956, v123, 502.

11. Toolan, H. W., PROC. SOC. EXP. BIOL. AND MED., 1951, v77, 572.

12. Suskind, R. G., Huebner, R. J., Rowe, W. P., and Love, R., *ibid.,* 1957, v94, 309.

13. Westwood, J. C. N., Macpherson, I. A., and Titmus, D. H. J., *Brit. J. Exp. Path.,* 1957, v38, 138.

Received September 13, 1957. P.S.E.B.M., 1957, v96.

Effect of Glucagon on Gastric Secretion. (23525)

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Zollinger and Ellison(1) recently have described a group of patients with progressive recurrent benign peptic ulcerations of the duodenum and upper jejunum associated with non-beta cell (presumably alpha cell) adenomas of the pancreas. These patients characteristically have a very high rate of gastric secretion and resistance to medical and surgical therapy. Although some of these patients have coexisting multiple adenomatosis of other endocrine glands(2) there is no clinical or laboratory evidence of hyperinsulinism. Since glucagon is secreted by pancreatic islet alpha cells which resemble the tumor cell type, Zollinger and Ellison suggested that glucagon might be the ulcerogenic factor. This study was designed to determine the effect of glucagon on gastric motility and secretion and thus to investigate(3) the likelihood of this substance being of pathogenic significance in the peptic ulceration associated with these bizarre pancreatic tumors.

Materials and methods. Gastric secretion: Nine adult males (age 29 to 59 years) were used; 4 of these patients had proven duodenal ulcer; 3 were diagnosed as psychophysiologic gastrointestinal reactions; and the remaining 2 patients were hospitalized for minor surgical problems unrelated to the gastrointestinal tract. Soon after awakening in the morning after a 12-hour fast, the patient was placed at bed rest for approximately 30 minutes before the experiment was begun. A No. 16 Levine tube was placed in the stomach and connected to a constant source of suction (100 mm Hg) with an intervening trap for collection of gastric secretions. The stomach was emptied and the initial specimen discarded. Gastric aspiration specimens were collected at 15-minute intervals during a one-hour control period, at the conclusion of which 1.5 mg of glucagon* in 10 cc of saline was rapidly injected by way of a previously established intravenous saline infusion in the forearm, and collections continued as before. The test period was continued for a maximum of 2 hours longer, or until the post glucagon infusion secretory rate approached the pre-infusion control. Venous blood samples were drawn without stasis from an indwelling needle in the antecubital vein and analyzed in duplicate. Capillary blood was drawn from a finger puncture. Glucose concentrations were determined by the Nelson-Somogyi method(4). The volume and free hydrochloric acid content was determined on each 15-minute sample(5). Gastric motility studies were performed on a series of 8 patients, 4 of whom had proven duodenal ulcers, the other 4 serving as normal controls. Motility was measured by an intragastric balloon attached to a recording device as designed by Hlad. As with the secretory studies, a one-hour pre-glucagon injection period was

* Kindly supplied by Eli Lilly Co., Indianapolis, Ind.

TABLE I. Effect of Glucagon on Gastric Secretion.

	Mean gastric secretory vol (ml/min.)		Mean HCl secretory rate (meq/min.)		Arterio-venous difference (mg %)	
	Control	Following glucagon	Control	Following glucagon	Control	Following glucagon
1*	26	8	.04	.02	4	13
2*	40	30	.5	.12	3	12
3*	29	18	.03	0	3.5	13.5
4	14	7	.02	0	4	9
5*	21	4	.03	.004	3	3
6	14	7	0	0	2.5	3
7	8	2.5	.02	.004	7	9
8	7	4	.01	.002	1	18
9	34	13	0	0	4	20

* Proven peptic ulcer.

used for the baseline control.

Results. The effect of intravenous administration of glucagon on the gastric secretory volume and its hydrochloric acid content is demonstrated in Table I and a typical experiment shown in Fig. 1. Following glucagon infusion there was a diminution in the total volume of gastric secretion; (mean 57%, range 25%-85%); and in the total and free hydrochloric acid fractions (mean 82%; range 50%-100%). In each instance gastric motility diminished following administration of intravenous glucagon. Both the diminution in gastric motility and the secretory changes occurred at the time of maximum arteriovenous blood glucose differences, but there was no definite correlation between the degree of peripheral glucose utilization and the degree of gastric response. The maximum response routinely occurred during the 30 minutes immediately following glucagon administration and in no instance did it persist for more than ninety minutes. There was no evidence of a later rebound response as described by Poth(6).

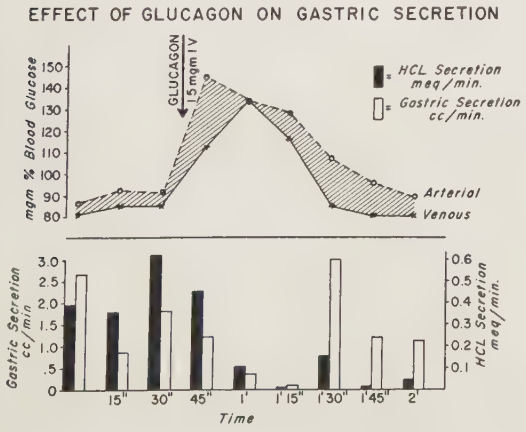

FIG. 1.

Discussion. The ulcerogenic properties of pancreatic secretion have been under investigation for many years. Complete diversion of pancreatic secretions by fistula from the gastrointestinal tract(7), and pancreatic duct ligation(8,9) both produce peptic ulceration, presumably by the removal of the alkaline pancreatic juice. Since total pancreatectomy does not result in ulceration, some pancreatic hormonal influence apparently is of importance in protecting against ulceration.

Hyperinsulinism, either from tumor or excessive administration, results in hypersecretion of HCl, and ulceration presumably due to the resultant hypoglycemia(10). Contrariwise, hyperglycemia in diabetes has been associated with a diminished incidence of peptic ulceration(11). Stunkard and Van Itallie(3) suggest that this effect is more intimately related to the glucose a-v difference (*i.e.*, utilization) than to the absolute blood glucose concentrations.

These previous studies(12) and the data presented in our experiments, suggest that the hyperglycemia and the increased glucose a-v difference(13) produced by glucagon results in diminished gastric motility, diminished total gastric secretory volume, and diminished hydrochloric acid secretion, activities that are associated with anti-ulcerogenic properties.

It seems unlikely, therefore, that the ulcerogenic factor in non-insulin producing adenomas of the pancreatic islet cells is glucagon. Such ulceration may result from an endocrine dysfunction related to the co-existing multiple adenomatosis, or to some as yet undiscovered pancreatic hormone.

Summary. 1. Diminution in gastric motility, gastric secretory rate, and hydrochloric acid production has resulted from glucagon administration in 17 patients. 2. These antiulcerogenic properties suggest that some factor other than glucagon is responsible for the severe peptic ulcerations commonly associated with non-insulin producing tumors of the pancreatic islets.

1. Zollinger, R. M., and Ellison, E. H., *Ann. Surg.*, 1955, v14, 709.

2. Eiseman, B., and Maynard, R., *Gastroenterology*, 1956, v31, 296.

3. Stunkard, A. S., Van Itallie, T. B., and Reiss, B. B., PROC. SOC. EXP. BIOL. AND MED., 1955, v89, 258.

4. Nelson, N., *J. Biol. Chem.*, 1944, v153, 375.

5. Todd, J. C., and Sanford, A. H., *Clinical Diagnosis by Laboratory Methods*, W. B. Saunders Co., Phila., 10th Ed., 1945, 455.

6. Poth, E. J., Fromm, S. M., DeYoung, R., and Aldrige, M., PROC. SOC. EXP. BIOL. AND MED., 1950, v74, 514.

7. Dragstedt, L. R., Montgomery, M. L., and Ellis, J. C., *ibid.*, 1930, v28, 109.

8. Elman, R., and Hartman, A. F., *Arch. Surg.*, 1931, v23, 1030.

9. Poth, E. J., Manhoff, L. J., Jr., and DeLoach, A. W., *Surgery*, 1948, v24, 62.

10. Poth, E. J., and Fromm, S. M., *Gastroenterology*, 1950, v16, 490.

11. Rotherburg, and Treicher, *Am. J. Dig. Dis.*, 1938, v5, 663.

12. Earle, A. S., Cahill, G. F., and Hoar, C. S., *Ann. Surg.*, 1957, v146, 124.

13. Elrick, H., Hlad, C. J., Jr., Arai, Y., and Smith, A., *J. Clin. Invest.*, 1956, v25, 757.

Received September 13, 1957. P.S.E.B.M., 1957, v96.

Development of Tolerance to Depressant Effects of Atropine on Isolated Rabbit Ileum.* (23526)

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The development of acquired resistance (tolerance) to the depressant action of atropine on gastrointestinal motility has been observed in man and in the dog(1,2). While suggestion of a related phenomenon in the isolated rabbit intestine can be found in the literature(3-5), a clear, controlled demonstration of the development of such tolerance *in vitro* has not been made. The work reported below is limited to studies of the depressant effects of atropine sulfate (atropine) and adiphenine hydrochloride† (adiphenine) on the amplitude of spontaneous contraction of the longitudinal muscle of isolated rabbit ileum. The results indicate that in this preparation tolerance to the effect of atropine is a reproducible and reversible phenomenon. Similar tolerance to adiphenine does not develop.

Methods. Sections of rabbit ileum were cut in lengths of 2 to 3 cm and mounted according to the classical method of Magnus in a bath containing 100 ml of oxygenated Tyrode's solution. Recordings of spontaneous contractions were effected electrically on a kymograph, the recording current being automatically shut off 10 sec of every minute. Contractions were recorded continuously for 5 min before and for 10 min after each addition of drug to the bath solution. At the end of these 10 min the bathing fluid was replaced with fresh Tyrode's solution. Second and third replacements followed at 2 min intervals. The ileum was then considered to be washed.

* This work was supported in part by Squibb Institute for Medical Research, and Smith, Kline and French Fn.

† Adiphenine hydrochloride = diethylaminoethyl diphenylacetate hydrochloride (Trasentine hydrochloride: Ciba).

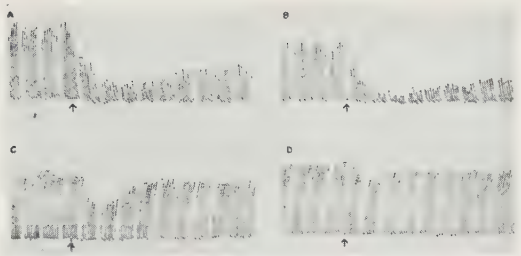

FIG. 1. Development of tolerance to atropine (40 μg%). Tracing A illustrates effect of first application of atropine; B, the second; C, the fifth; and D, the seventh. Time of application is indicated by the arrow.

This overall procedure was usually repeated every 30 min over a period of 3 to 5 hr. Concentrations of the drugs were expressed as mg or μg of the salt per 100 ml of bath solution (mg% or μg%).

Results. Atropine Tolerance. Repeated application of atropine (40 μg%) at 30 min intervals as described above invariably resulted in the development of tolerance, such that the sixth or seventh application produced little, if any, depression of the height of spontaneous contraction A typical experiment is shown in Fig. 1. Studies of 16 sections of ileum are summarized in Fig. 2. Curve B represents the average response to the first application of the above concentration of atropine, while curve C represents the average response to the sixth application. When once developed, tolerance persisted with continued periodic administration of atropine over the total course of 5 hr (10 applications of drug). The possibility that atropine tolerance was produced solely by the repeated washings of the ileum was investigated in the following experiment. Eleven sections of ileum were subjected to washings every 30 min for 2½ hr. Atropine (40 μg%) was then applied for the first time. Curve A in Fig. 2 shows the average depression of height of spontaneous contractions produced by this application.

Evidence for Accumulation of Atropine. The degree of spasm induced by 10 μg% acetylcholine chloride (Ach) was determined for each of 10 sections of ileum. After 10 min of exposure the sections of ileum were washed. Tolerance to atropine was then developed by the usual procedure *viz.*, 5 applications of atropine with each application followed 10 min later by the usual washing. After the final washing the degree of spasm induced by the same concentration of Ach was again determined. The development of tolerance reduced the average spasmodic response to Ach by approximately 75% of its control value. Inasmuch as this response to Ach does not change with semi-hourly applications and washings of the drug over 3 hr, the observed reduction is probably referable to incomplete removal of atropine by the washing process and a resultant accumulation of atropine. In 9 sections of ileum tolerance to atropine was developed by the usual procedure. The preparations were subsequently washed at 30 min intervals for 2½ hr, after which time atropine was again applied. The average results, shown in figure 3, support the probability of an association between the phenomenon of atropine tolerance and the accumulation of atropine within the tissues.

Adiphenine. Experiments were performed to investigate the possible occurrence of a similar type of tolerance in response to repeated applications of adiphenine. Sections of ileum were repeatedly treated with 1 mg% adiphenine in a manner similar to that described for atropine. Fig. 4 (upper tracing) illustrates the average reductions in height of spontaneous contractions in response to the first (solid line) and sixth (broken line) applications of adiphenine to 9 sections of ileum.

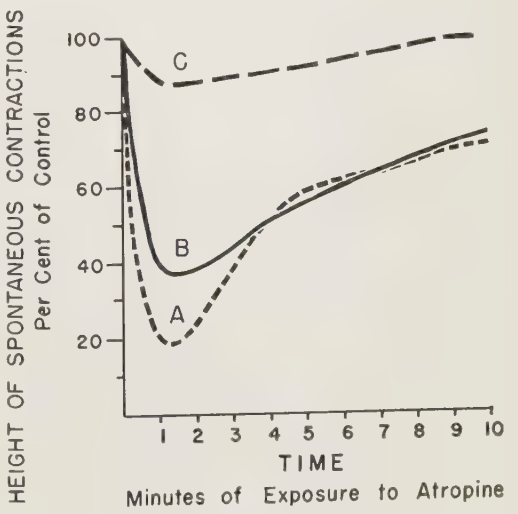

FIG. 2. Effect of washings on response of the rabbit ileum to atropine. See text.

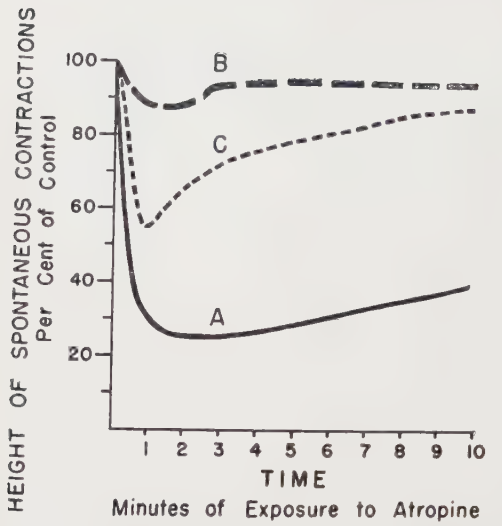

FIG. 3. Effect of washings on previously developed tolerance to atropine. Curve A represents average response to first application of atropine; B, the fifth; and C, the sixth. Between the fifth and sixth applications the ileum was washed at 30 min intervals for 2½ hr.

In no experiment was there any evidence of the beginning of the development of tolerance even over a time period of 5 hr. Moreover, when this concentration of adiphenine was applied to ileum which had been rendered completely tolerant to atropine, the depression was as great as it was in non-tolerant ileum. Fig. 4 (lower tracing) shows the average results of 4 experiments in which (a) 1 mg% adiphenine was applied to fresh ileum (solid line), (b) the ileum was made tolerant to atropine by the usual procedure, and (c) 1 mg% adiphenine was again applied (broken line).

Discussion. The results reported in this paper demonstrate the occurrence, under certain conditions, of a type of motility of smooth muscle, which is refractory to atropine. While the nature of the mechanisms responsible for this phenomenon are at present unknown, the phenomenon itself may be of great practical value. Hitherto, the degree of antispasmodic activity of an agent has been expressed in terms of its proportion to the degree of activity of atropine, papaverine and/or pyribenzamine. Adiphenine, for example, has been described as possessing low atropine-like activity as determined by its ability to reduce spasms induced by acetylcholine. On the other hand, because of its relatively high activity in depressing spasms induced by barium chloride, adiphenine is usually classified as a papaverine-like compound. Controversial data, concerned with the mode of action of barium chloride itself(6,7) further complicate this classification. The results presented in this paper reveal a clearly demonstrable difference between the actions of adiphenine and atropine on a physiological entity, *viz.*, amplitude of spontaneous contraction. Investigations of other antispasmodic agents on this preparation, with particular regard to their tendency to develop tolerance and to their action on atropine tolerant motility, would contribute significantly to the elucidation of true similarities and differences in antispasmodic activity.

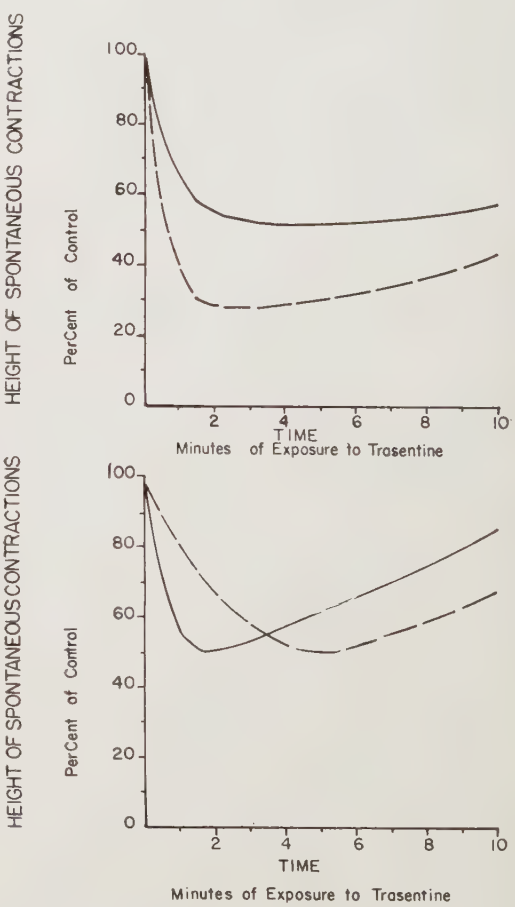

FIG. 4. Upper tracing: depressant effects of adiphenine (Trasentine) (1 mg%). Lower tracing: effect of adiphenine on atropine tolerant motility.

Summary. 1) Repeated exposure of isolated sections of rabbit ileum to atropine (40 μg%), with washings 10 min after each exposure, results in development of tolerance, such that by sixth or seventh exposure, the depressant effects of atropine on height of spontaneous contraction are minimal or even abolished. The phenomenon of tolerance appears to be associated with gradual accumulation of atropine within the tissue. 2) Repeated exposure of rabbit ileum to adiphenine (1 mg%) results in no observable tendency toward the development of tolerance. Moreover, when adiphenine is applied to ileum which previously has been made tolerant to atropine, adiphenine is fully effective in depressing the height of spontaneous contraction.

1. Gray, G. W., and Seevers, M. H., *J. Pharm. and Exp. Ther.*, 1955, v113, 319.

2. Quigley, J. P., PROC. SOC. EXP. BIOL. AND MED., 1937, v36, 450.

3. Chang, P. Y., and Hsu, F. Y., *Quart. J. Exp. Physiol.*, 1942, v31, 299.

4. Youmans, W. B., *Nervous and Neurohumoral Regulation of Intestinal Motility*. Interscience Publishers, N. Y., 1949.

5. Feldberg, W., and Solandt, O. M., *J. Physiol.*, 1942, v101, 137.

6. Ambache, N., *ibid.*, 1949, v110, 164.

7. Feldberg, W., *ibid.*, 1951, v113, 483.

Received September 16, 1957. P.S.E.B.M., 1957, v96.

A Kwashiorkor-Like Syndrome Observed in Monkeys Fed Maize.* (23527)

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To understand all aspects of the pathogenesis of a particular disease in man, one should be able to reproduce it in the experimental animal. This has been eminently true in the evolution of our knowledge of certain human nutritional disease syndromes, such as beriberi, scurvy, rickets and pellagra; for great advances were made in an elucidation of the biochemical and anatomical alterations in these disease states when they could be studied in the laboratory. In contrast, our woeful ignorance of the pathogenesis of such diseases as endemic goiter and, until recently, pernicious anemia stems from the fact that the physiologic and morphologic pattern has not yet been produced in experimental animals. Today, kwashiorkor has world-wide distribution and a high prevalence. Although clinical studies, particularly those dealing with therapy, have revealed something of its pathogenesis, a more basic understanding is likely to be obtained if the disease can be reproduced in the laboratory.

Kwashiorkor is one of a group of several syndromes which occur endemically and which are associated in many parts of the world with an intake of virtually a single foodstuff. In the case of kwashiorkor this foodstuff is maize. Many years ago corn was shown to be deficient in certain inorganic elements, amino acids and vitamins(1); hence, it is not surprising that a multiple disease syndrome might follow extended use of this grain. Because investigations dealing with deficiencies of *single* essential nutrients have now furnished a baseline of biochemical and anatomical changes which may occur in many animal species, the time appears favorable to renew the study of *multiple* deficiency states such as those which may be produced by feeding single natural foodstuffs. We have recently undertaken such a program in this laboratory in an attempt to reproduce in experimental animals certain nutritional diseases which occur naturally in man and whose pathogenesis is not clear. In the present com-

* Portions of data presented herein were supported by grants in aid from Amer. Cancer Soc. to the Committee on Growth and Division of Research Grants, U. S. Public Health Service (H-2999).

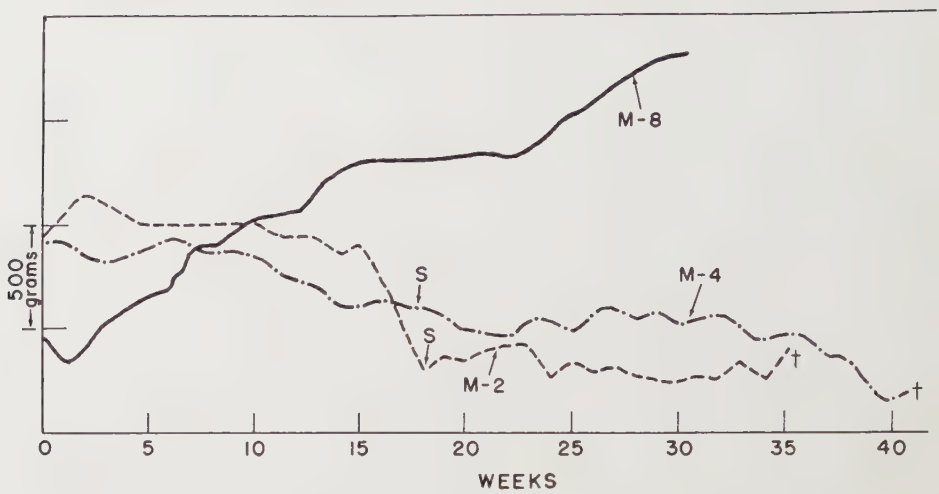

FIG. 1. Growth curves of 2 animals fed maize (M-2 and M-4) and a control (M-8).

munication we wish to describe a syndrome having many of the characteristics of kwashiorkor which has been observed to develop in monkeys fed maize.

Materials and methods. Twelve monkeys (*Cercopithecus griseoviridis* or *aethiops*) have been under study to date. All were young growing male or female specimens whose exact age was unknown, though each was in the process of acquiring its second dentition. They were housed in a well-ventilated, constant temperature, windowless room in stainless steel cages having coarse screen bottoms over cedarwood shavings. Tap water was allowed *ad libitum*. For 8, the basic foodstuff was ground unenriched yellow cornmeal (Wilkins Rogers Milling Co., Washington, D.C.) which was made into mush with water and fed raw or as cakes after heating in the oven. On analysis the corn contained per 100 g dry weight: protein, 10.2; lipid, 7.6; ash, 1.04; calcium, 55 mg; phosphorus, 215 mg; ascorbic acid,† .72 mg; thiamine,† .50 mg; riboflavin,† .18 mg; and vit. A,† 230 I.U. No other foods were given save occasional lumps of sugar and ascorbic acid, as noted below. One animal received an adequate commercial ration supplemented with sources of vit. C. Three monkeys, used in another study were fed rice supplemented with ascorbic acid and were utilized as inanition controls since their weights paralleled those of the group fed maize. Animals were fed and watered every day and weighed weekly. Blood for chemical determinations was withdrawn from the femoral vein. Liver biopsies were performed aseptically under local anesthesia with a Vim-Silverman needle. Total lipid in liver tissue was determined by ethanol-ether extraction. Complete autopsies were performed when animals died. Microscopic sections of all organs were made by routine technics. Microradiographs of ground sections of bone were made with Phillips C.M.R. equipment.

Results. After being placed on the diet a gradual loss of weight occurred. As shown in Fig. 1 for 2 representative animals, this loss was not excessive: a total of 800 and 600 g in 35 and 41 weeks per animal, when initial weights were approximately 2350 g. In contrast, the control gained 1500 g in a similar period. During the 18th week both animals shown in Fig. 1 exhibited clinical scurvy: loosening of the teeth, bleeding gums, and weakness of the lower extremities. One developed small subcutaneous hemorrhages over the surfaces of the arms and legs. Ascorbic acid (100 mg) was administered at this time, with complete amelioration of these signs. Vit. C therapy was continued at bi-weekly intervals and has been administered to all

† These analyses were performed through the courtesy of Major R. A. Huseby, M. C., Medical Nutrition Laboratory, Denver, Colo.

maize-fed animals since that time.

As the deficient state progressed, the animals became listless and apathetic. They could be handled without difficulty, making

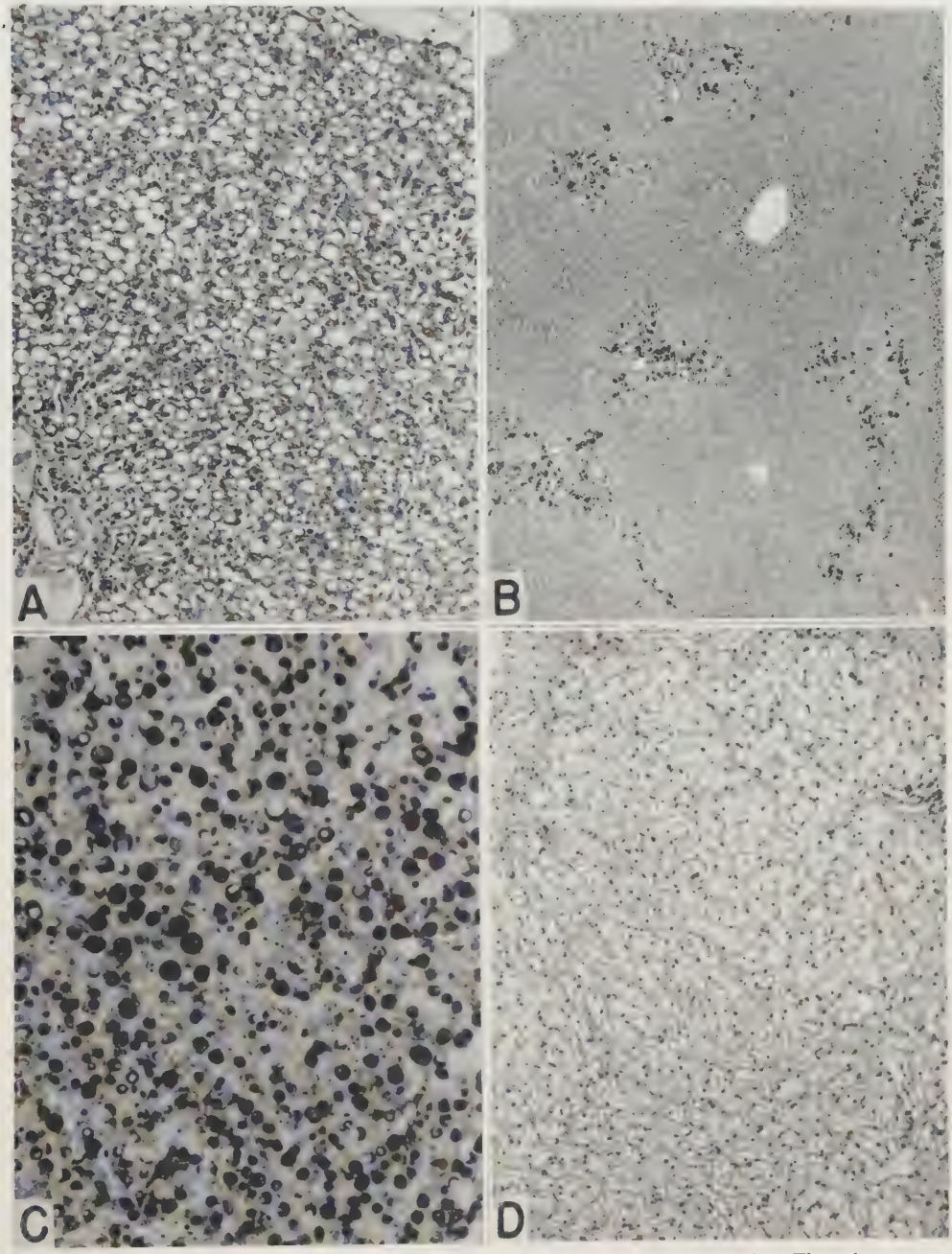

FIG. 2. A. Section from liver of monkey M-4 dying after 41 weeks on corn diet. There is extreme diffuse fatty infiltration (H. and E., × 90). B. Section from liver of animal autopsied after 13 weeks on maize diet. Fat is present only in cells of peripheral (periportal) portions of liver lobules. Note 2 central veins in this field (oil red O, × 35). C. Section from liver of animal (M-2) dying after 35 weeks to show extensive fatty change (oil red O, × 100). D. Biopsy of liver from animal (M-9) which has been on maize regimen for 14 weeks. There is periportal fatty change (H. and E., × 100).

TABLE I. Liver Lipid and Plasma Protein Concentrations.

No.	Weeks on diet	% liver lipid	Total	Alb.	Glob.
4	41 M*	22.8	3.53	.95	2.58
2	35 M	15.0	3.87	1.16	2.71
13	7 M	5.1	6.0		
8	0		6.80	4.01	2.79
12	0		7.05		
10	0		6.8		
1	0	4.2			
5	34 R*	3.9	6.9		

* M = Maize diet; R = Rice diet.

no attempt to retreat to the rear of the cage. They assumed a characteristic pose, sitting in the corner of the cage with head bent forward between front and hind limbs so that it almost touched the floor. Only when *in extremis* did the animals lie on their sides. Periorbital edema was noted in a female after 26 weeks of the deficiency. Examination at this time revealed edema about the external genitalia. Loss of hair over the top of the head was seen at the time edema became manifest in this animal. No loss of hair has been observed in other monkeys, nor has there been any change in color of the coat.

In 3 animals which have come to autopsy, the most prominent finding has been an increase of fat in the liver by chemical analysis (Table I). In the animal living longest (41 weeks) the fatty change is extreme. Less fat is present in the liver of a monkey living for 35 weeks and least in one dying after 7 weeks on the corn diet. Histological examination of the liver confirms the chemical findings. In the most severely affected livers all cells are distended with vacuoles which in frozen sections stained with oil red O prove to be lipid (Fig. 2). In such livers the distribution of fat is uniform throughout. Studies of earlier stages either at autopsy or on biopsy indicate that fat accumulation appears first in the periportal regions of the lobules and then spreads to involve the remainder of these structures. In 2 animals dying after 35 and 41 weeks on the diet iron pigment is present in large amounts within the liver cells. No inflammatory cells nor any increase in reticulum fibers have been noted.

Other alterations which have been found on microscopic examination are atrophy of intestinal glands, loss of lipids in the adrenal cortex and arrest in growth of the bones. No changes have been found in the pancreas. In the colon there is cystic dilatation of the mucosal glands whose lining cells are flattened. Cross sections of the cortices of the long bones reveal failure of the normal primary bone to be replaced by osteons (Fig. 3). Hence the skeletal tissue is more immature than it should be at this age. No evidence of osteomalacia is present.

As shown in Table I, concentrations of total plasma proteins were reduced in the 2 animals dying in the late stages of the deficiency. These monkeys had high fat concentrations in the liver. Periportal fat accumulation was beginning in the animal (M-13) sacrificed after 7 weeks, at which time the plasma protein concentration was not particularly reduced. Current biopsy studies of the liver indicate that fatty infiltration antedates changes in plasma protein concentrations.

Discussion. As it is observed in various parts of the world today, the kwashiorkor syndrome may be characterized as follows: 1) Retardation of growth, 2) Weight loss with muscular wasting, 3) Apathy and other psychic changes, 4) Dermatitis and pigmentary alterations in the hair, 5) Edema with hypoalbuminemia, 6) Enlargement of the liver as a result of fatty infiltration which begins in the periportal areas, and 7) Gastrointestinal disturbances(2). The disease is seen in children who have been weaned to a diet which is poor with respect to both quantity and quality of protein. A corn diet furnishes insufficient protein, which is deficient in at least 2 amino acids: tryptophan and lysine, and in which there may be imbalances in amino acid content as well(3). Since the early studies of McCollum(1) on the feeding of cereal grains to non-ruminants, corn has often been used in rations which were employed to study nutritional disease. For instance, the experimental production of rickets(4) in rats and blacktongue in dogs(5) depended on a high proportion of corn in the dietary regimens. Recently interest has been reawakened in corn because of its presence in large amounts in the diet in areas where kwashiorkor is endemic. Studies dealing with the inadequacies

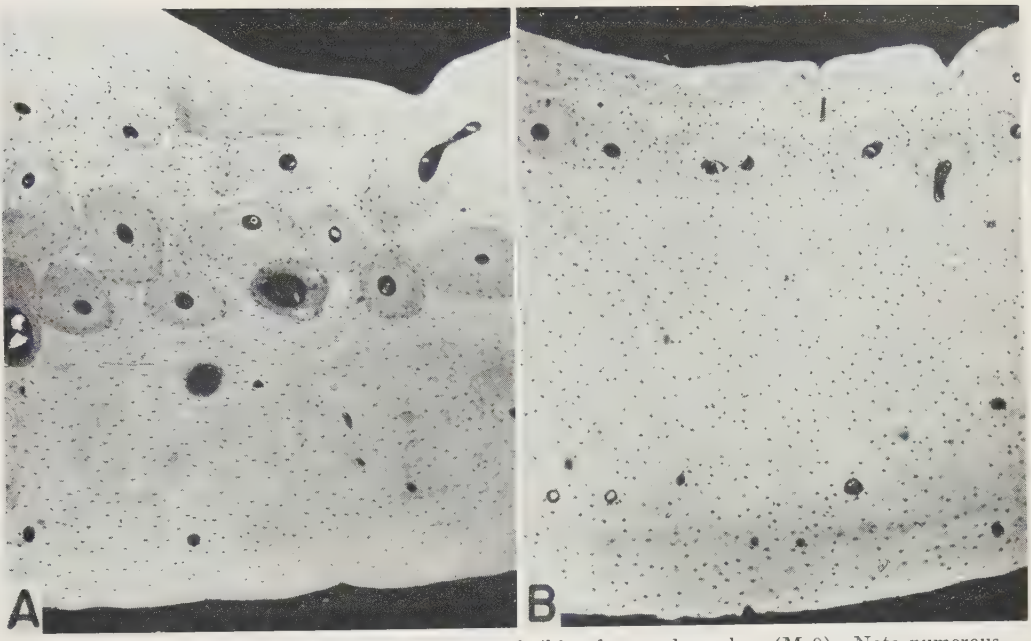

FIG. 3. A. Microradiogram of anterior cortex of tibia of normal monkey (M-8). Note numerous Haversian systems replacing the membranous bone. B. Cortex from same area of tibia of animal (M-2) dying after 35 weeks on corn. There had been no growth (see Fig. 1). Only a few osteones have appeared. There is no evidence of osteomalacia (both, × 50).

of this cereal grain, particularly with respect to protein have been reported(6,7,8,9). Such investigations have dealt primarily with growth studies in rats. However, Shils and his co-workers(8) have examined the livers of rats fed corn diets; in these animals periportal fatty infiltration has been observed. In all the studies just referred to save those of Gillman *et al.*(9) the corn rations have been supplemented by minerals and vitamins. The latter investigators utilized corn diets supplemented only with fermented milk and observed fatty livers which developed scarring. In our experience rats which have been fed a completely unsupplemented corn diet exhibit an entirely different disease syndrome(10) than that which has just been described in the monkey; hence it is felt that the rat is not a suitable species with which to work.

The experiments herein recorded have now been in progress for some time. Chemical studies, serial biopsies of animals now under observation, as well as the autopsy findings already mentioned, all appear to indicate that a syndrome rather similar to kwashiorkor can be produced in monkeys. Obviously the problem is a long term one, but in view of these promising findings it is felt this report is warranted in hopes that others may begin to study this syndrome.

Summary. Growing monkeys have been maintained up to 41 weeks on a diet of maize. Animals exhibit growth failure, loss of weight, weakness, apathy, edema with hypoalbuminemia and fat accumulation in the liver, which begins periportally and then spreads to involve the entire lobule. This syndrome appears to have many of the characteristics of kwashiorkor in children.

1. McCollum, E. V., Simmonds, N., and Pitz, W., *J. Biol. Chem.*, 1916, v28, 153.

2. Jeliffe, D. B., *Infant Nutrition in the Subtropics and Tropics, W. H. O. Monogr. Ser.* No. 29, Geneva, 1955.

3. Maize and Maize Diets, F. A. O., Nutritional Studies, No. 9, Rome, 1953.

4. McCollum, E. V., Simmonds, N., Shipley, P. G., and Park, E. A., *Am. J. Hyg.*, 1921, v i, 492.

5. Goldberger, J., and Wheeler, G. A., *Pub. Health Rep.*, 1928, v43, 172.

6. Sauberlick, H. E., Chang, W-Y., and Salmon, W. D., *J. Nutrition*, 1953, v51, 623.

7. Hogan, A. G., Gillespie, G. T., Kocturk, O., O'dell, B. L., and Flynn, L. M., *ibid.*, 1955, v57, 225.

8. Shils, M. E., DeGiovanni, R., and Stewart, W. B., *ibid.*, 1955, v56, 95.

9. Gillman, J., Gillman, T., Mandelstam, J., and Gilbert, C., *Brit. J. Exp. Path.*, 1945, v26, 67.

10. Follis, R. H., Jr., *Fed. Proc.* 1957, v16, 356.

Received September 16, 1957. P.S.E.B.M., 1957, v96.

Anticoagulant, Lipemia Clearing and Other Effects of Anionic Polysaccharides Extracted from Seaweed. (23528)

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Recent investigations have been made of the effects of heparin-like compounds on blood coagulation and lipemia clearance(1,2). This paper describes the hematologic and other responses to a number of such compounds, several of which have not been previously studied. These materials were prepared from aqueous extracts of red seaweeds, and are galactosans of varying degrees of sulphation.

Materials and methods. Carrageenan, a sulphated galactosan, and 6 other sulphated polysaccharides were studied.* Hyaluronate (3) and the chondroitin sulphates A(4) and B(5) were prepared using previously described methods. Measurements of lipemia were performed by reading directly the optical density of plasma in 12 x 70 mm cuvettes at 650 mμ in a Coleman Jr. spectrophotometer. Clotting times were determined in a series of small tubes ¼″ in diameter containing 1 ml whole blood at 27°C. The tubes were inspected for coagulation every ½ minute. The endpoint was taken as the time when a tube could be placed on its side without any corresponding shift in blood level. Differential cell counts were done in triplicate. The hematoxylin-eosin and Hotchkiss-McManus periodic acid Schiff stains were done in the usual manner. Reducing activity was measured by a modification of the Park and Johnson procedure(6).

Twelve dogs were fed triolein (4 g/kg) after an overnight fast. The optical density of the plasma of all fasted animals was initially less than 0.09. Water was permitted *ad lib.* In 45 determinations on 12 dogs, all plasma turbidities two hours after feeding were within the range of 0.24 to 0.48 optical density unit (mean 0.41 ± 0.05). Immediately after removing 6-7 ml of blood 2 hours after the fat meal, the various polysaccharides prepared in a concentration of 2 mg/ml of saline were injected intravenously at a dose of 1 mg/kg body weight. Fifteen minutes after injection, a second blood sample was obtained. Blood was withdrawn in clean syringes and immediately added to tubes containing dry heparin. Samples of this blood were placed in clotting time tubes.

An aqueous solution of sodium heparinate was prepared containing 10 mg/ml and diluted with an equal volume of 0.3 N NaOH. After boiling this mixture for 15 minutes, the solution was cooled and 2 volumes of ethanol were added. The precipitate was removed by centrifugation and washed repeatedly with ethanol. This material demonstrated a threefold increase in reducing activity. A similar heparin solution was prepared using 6.0 N NaOH. The reducing activity increased some 15-fold and the molecule was partially desulphated. These materials were termed heparin A (normal), B (0.30 N NaOH), and C (6.0 N NaOH).

The seaweed extracts were purified by dissolving the commercial grade material in hot water and precipitating with isopropanol.

Results. The anticoagulant and lipemia clearance activities of the compounds described above were determined in triplicate.

* Seaplant Corp. of New Bedford, Mass., through the courtesy of Leonard Stoloff.

TABLE I. Effects of Some Anionic Polysaccharides on Lipemia Clearance and Clotting Time in the Dog.
(All figures represent means of measurements obtained in 3 dogs.)

Polysaccharide	Turbidity (O.D. at 650 mμ)	Clotting time (min.)	Comments
None	.30-.48	5-10	
Hyaluronate	.35	5	
Chondroitin sulphate A	.30	6.5	
B	.25	18	
Heparin A	.09	45	
B	.10	40	
C	.10	15	
Carrageenan	.44	5.5	dr* l
Extracts of:			
1) Furcellaria festigiate	.38	5.5	dr l
2) Eucheuma spinosum	.39	.12	de
3) *Gigertina acicularis*	.40	50	de
4) " *pistillate*	.35	6	de
5) " *radula*	.36	6	de
6) Iridaea	.40	5	de

* dr = drowsiness, l = lethargy, de = defecation.

Results are given in Table I. The range of response within any given trio of dogs was small. All dogs given the seaweed polysaccharides displayed yawning and lethargy, and fell asleep for about 10 minutes immediately after injection. Most of these animals also defecated immediately. The lethargy response occurred in over a dozen different dogs, while the anticoagulant activity of extract #3 (*Gigertina acicularis*) was not found in 2 of this group of 12 animals.

Unlike heparin, the anticoagulant activity of extract #3 increased with time, and was still evident 24 hours after injection. This change in activity with time was studied in 3 animals given extract #3 (1 mg/kg). Differential cell counts were determined in duplicate on each animal as well as the hematocrit and clotting time at ½, 1, 2, 4, and 24 hours after injection. During this time there were no changes in rectal temperature nor was any evidence of hemolysis observed. The anticoagulant activity was maximal after 4 hours, while none of the changes in the differential cell count or hematocrit were significantly different from control levels.

Three dogs were injected with 15 mg/kg of this polysaccharide (from *Gigertina acicularis*). Arterial and venous pressures and electrocardiograms of 2 of these were continuously recorded. The small changes that occurred were similar to those produced by equivalent amounts of 5% gelatin solutions. Both dogs died within 24 hours. The survivor was sacrificed at the same time. The tissues of these animals were fixed in formalin, sectioned and stained in duplicate with H. & E. and PAS.

Microscopically, all organs showed changes of venous congestion. The macrophages of the spleen and liver were strongly stained by PAS, indicating a powerful affinity of the polysaccharide for the reticulo-endothelial system.

The most remarkable changes were evident in the kidneys. The glomerular tufts had taken on the appearance seen in membranous glomerulonephritis. The basement membrane was strikingly thickened, and the glomerular epithelium showed vacuolation.

Discussion. These experiments were designed using a large amount of triolein (olive oil) to produce marked plasma turbidity. Under these conditions lipemia clearance will only occur when a very active compound is used. The alkaline degradation products of heparin were quite active in this respect, although the more degraded material lost its anticoagulation activity. The observation has been reported by Zollner and co-workers (7).

Extract #3 is a very potent anticoagulant. The mechanism of this anticoagulant activity is currently under study. The profound pathological changes in the kidney that occur with large doses of the polysaccharide give the appearance of being a direct reaction with some component of glomerular membrane or an extreme local alteration in osmotic equilibrium. Popper *et al.*(8) have shown that intravenous pectin infusions caused a dilation of the renal tubules and glomerular spaces, as well as a deposition of material that resembles amyloidosis. These effects were also seen with extract #3. The renal function of animals so treated is under study.

Summary. A number of anionic polysaccharides were investigated with respect to their anticoagulant and lipemia clearing activity. Only heparin was effective in clearing

the massive lipemia used in this study. This effect persisted even when the anticoagulant activity was destroyed by alkaline degradation. Another potent anticoagulant was found in a highly sulphated galactosan isolated from seaweed. This compound produced extensive pathologic changes in the kidney when administered in large doses.

The authors wish to gratefully acknowledge the guidance of Dr. John Rose and Dr. Roger Baker of this Hospital.

1. Arge, E., *Acta Med. Scand.*, 1956, v155, 496.

2. Seifter, J., and Bader, D. H., PROC. SOC. EXP. BIOL. AND MED., 1954, v86, 709.

3. Alburn, H., and Williams, C. E., *Ann. N. Y. Acad. Sci.*, 1950, v53, 971.

4. Einbinder, J., and Schubert, M., *J. Biol. Chem.*, 1950, v185, 725.

5. Meyer, K., and Chafee, D. B., *ibid.*, 1941, v138, 491.

6. Park, J., and Johnson, V., *ibid.*, 1949, v181, 149.

7. Zollner, N., Rothemund, E., and Seitz, W., *Klin. Wochschr.*, 1954, v32, 1096.

8. Popper, H., Wold, B., Meyer, K., Kozoll, D., and Steigman, F., *Arch. Surg.*, 1954, v50, 34.

Received September 5, 1957. P.S.E.B.M., 1957, v96.

Effect of Some Monosaccharides on Serum Inorganic Phosphate in the Normal Dog. (23529)

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Soon after the discovery of insulin, it was found that the hormone has a depressing effect upon serum inorganic phosphate(1). A similar effect was described after glucose administration(2,3). The action of glucose on serum inorganic phosphate appeared to be due to induced insulin secretion since it was not possible to elicit a normal response in the pancreatectomized dog(4), or in the alloxanized dog(5). It seemed of interest to study the effect of various monosaccharides upon serum inorganic phosphate. It has been mentioned that glucose, fructose and galactose show a depressing effect(6,7). It has been also demonstrated that fructose may decrease serum phosphate in the pancreatectomized dog (8). In this paper, the effects of 8 sugars and one polyalcohol (D-Sorbitol), independently injected by vein and in some cases mixed with glucose and/or insulin are reported.

Material and methods. Apparently normal dogs weighing between 12 and 20 kg, fed *ad libitum* on Purina Chow and twice weekly on meat, were used. The animals were previously trained by repeated venipunctures to avoid fear and anger reactions. When the same dog was used for several experiments, a previous test was carried out on a different day with glucose in order to check whether the animal showed a normal phosphate fall. The experiments were carried out after a fasting period of about 15 hours. A venous blood sample was taken, the sugar injected in 3M solution (1 ml/kg) within a period of one minute and new samples obtained at 15, 30, 45 and 60 minutes. D-Galactose was used in 1.5M concentration (2 ml/kg). When 2 sugars were administered, one ml of the 3M solution/kg of each was injected. Glucagon-free insulin (generously furnished by Eli Lilly Co.) was used by vein at the dose of 0.1 U/kg. Serum inorganic phosphate was determined in duplicate using the Fiske and Subbarow procedure(9) adapted to the photoelectric colorimeter. Changes in serum inorganic phosphate were expressed in terms of "Deltas" (differences from the initial values in mg/100 ml). The statistical significance of the results was appraised with the use of the "t" test, by comparison with the spontaneous variation observed by taking blood samples every 15′ up to 60′, under experimental conditions. The compounds used (C.P.) were:

* The author wishes to express his gratitude to Mr. Francisco Pena for his technical assistance.

TABLE I. Averages and Standard Errors of Changes in Serum Inorganic Phosphate in mg/100 ml (Deltas) after Venous Injection of Different Sugars.

	No. of exp.	Min. after injection 0′	15′	30′	45′	60′
D-Glucose	16	4.11 ± .23	−.26*± .05	−.42*± .07	−.42*± .05	−.23*± .08
D-Galactose	10	3.97 ± .17	−.10*± .05	−.22*± .05	−.23*± .07	−.20*± .06
D-Levulose	16	4.08 ± .20	−.09 ± .05	+.09 ± .06	+.30*± .06	+.38 ± .13
L-Rhamnose	6	3.99 ± .41	+.01 ± .06	−.08*± .03	−.05*± .04	−.06*± .06
D-Sorbitol	6	3.63 ± .20	−.44*± .15	−.40*± .08	−.21 ± .19	+.07 ± .88
L-Sorbose	6	4.21 ± .31	−.33*± .18	−.48*± .10	−.27*± .10	−.17*± .09
L-Arabinose	12	3.87 ± .24	+.07 ± .06	+.04 ± .06	+.04 ± .07	+.14 ± .07
D-Arabinose	10	3.83 ± .23	+.04 ± .06	+.20 ± .08	+.52*± .12	+.87 ± .10
D-Xylose	10	3.69 ± .16	−.11*± .03	−.14*± .04	−.20*± .07	−.20*± .05
Spontaneous variations	10	3.96 ± .26	−.10 ± .07	+.13 ± .07	+.10 ± .05	+.37 ± .13

* Statistically significant changes as compared to spontaneous variation; $t > 2$.
(Underlined)—Decreasing effect greater than .30 mg/100 ml.

D-Glucose,† D-Galactose,† D-Levulose (pyranose),‡ L-Rhamnose,‡ D-Sorbitol,§ L-Sorbose (Tech.),‡ L-Arabinose,‡ D-Arabinose‡ and D-Xylose.‡ The 3 pentoses were used alone, mixed with glucose, with insulin and with glucose + insulin.

Results are shown in Tables I and II.

Discussion. Previous work(5) has shown that a decrease in serum inorganic phosphate greater than 0.30 mg/100 ml occurred after glucose injection in normal dogs, as opposed to alloxanized dogs who show little or no response. We have then taken a decrease greater than 0.30 mg/100 ml from the initial level after administration of a sugar as showing a definite effect. Levine *et al.*(8) take as significant a decrease greater than 0.20 mg/100 ml. If the fasting levels of serum inorganic phosphate are taken to vary between 3 and 5 mg/100 ml and a 5% error in the method used is assumed, 0.15 to 0.25 mg/100 ml is the range of uncertainty to be expected in connection with the procedure.

D-Glucose, L-Sorbose and D-Sorbitol have a clear-cut decreasing effect on serum inorganic phosphate. However, statistical comparison with the spontaneous variation makes it appear that D-Galactose, L-Rhamnose and D-Xylose have some effect, apparently masked by the rise in serum phosphate observed after

TABLE II. Averages and Standard Errors of Changes of Serum Inorganic Phosphate in mg/100 (Deltas) Induced by Some Pentoses in Mixture with D-Glucose and/or Insulin.

	No. of exp.	0′	15′	30′	45′	60′
Insulin	6	3.93 ± .23	−.40 ± .07	−.76 ± .16	− .86 ± .15	− .66 ± .25
L-Arabinose + insulin	9	3.83 ± .25	−.26 ± .07	−.90 ± .12	−1.23 ± .16	−1.23 ± .25
D-Arabinose + "	8	3.75 ± .28	−.30 ± .10	−.55 ± .16	− .40†± .16	− .10 ± .24
D-Xylose + "	10	4.07 ± .11	−.32 ± .09	−.73 ± .18	−1.02 ± .22	−1.10 ± .20
D-Glucose	16	4.11 ± .23	−.26 ± .05	−.42 ± .07	− .42 ± .05	− .23 ± .08
L-Arabinose + D-Glucose	6	4.09 ± .33	−.14 ± .05	−.32 ± .10	− .17*± .08	+ .04*± .07
D-Arabinose + "	6	4.06 ± .40	−.01 ± .18	−.23 ± .17	+ .32*± .08	+ .62*± .12
D-Xylose + "	6	3.68 ± .23	−.37 ± .10	−.26 ± .14	− .27 ± .18	− .23 ± .14
D-Glucose + insulin	6	4.19 ± .32	−.74 ± .13	−.99 ± .09	−1.07 ± .16	−1.13 ± .13
L-Arabinose + D-Glucose + insulin	7	3.69 ± .22	−.47‡± .08	−.83 ± .09	−1.09 ± .19	−1.11 ± .23
D-Arabinose + " + "	6	3.83 ± .23	−.36‡± .09	−.43‡± .07	− .35‡± .12	− .30‡± .12
D-Xylose + " + "	6	3.70 ± .28	−.43 ± .10	−.89 ± .14	−1.06 ± .17	−1.26 ± .20

* Statistically significant modifications in comparison to changes induced by glucose.
† *Idem* insulin.
‡ " glucose + insulin.

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‡ Eastman Organic Chemicals.
§ Pfanstiehl Laboratories.

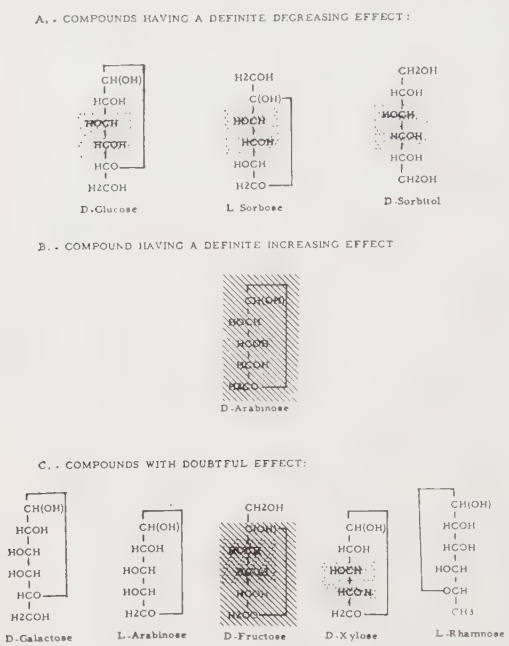

FIG. 1. Classification of various compounds according to their effect on serum inorganic phosphate.

successive blood extractions. Compared with the spontaneous variation, D-Fructose showed a rise at 45′ (Table I). Sixteen experiments with D-Fructose were carried out without being able to confirm previous work(6,7) showing a decreasing effect on serum phosphate. Levine *et al.*(8) reported a clear decrease induced by this sugar in pancreatectomized dogs but they did not carry out experiments in normal animals. The type of fructose used was not mentioned in these papers.

D-Arabinose, which causes serum phosphate to rise distinctly, inhibited almost completely the decrease induced by glucose and partially that produced by insulin or by mixture of glucose + insulin. L-Arabinose showed some inhibitory effect with respect to glucose effect at 15′ after glucose + insulin.

It appears that D-Glucose, D-Sorbitol and L-Sorbose produce a clear-cut decrease in serum inorganic phosphate and that D-Arabinose induces a rise.

By examining the chemical configuration of the substances used(10), (Fig. 1) it seems that the common feature of those exhibiting a definite decreasing effect is the arrangement of the OH groups in carbons 3 and 4 in the hexose molecule. D-Fructose has the same configuration in carbons 3 and 4, however its 5 last carbon atoms have the same structure as D-Arabinose, which has been shown to increase markedly the serum phosphate concentration. D-Xylose also possesses the above-mentioned configuration at the 3 and 4 carbon atoms but in a pentose rather than a hexose skeleton.

Summary. The effect of D-Glucose, D-Galactose, D-Fructose, L-Rhamnose, L-Sorbose, D-Sorbitol, D-Arabinose, L-Arabinose, and D-Xylose on the serum inorganic phosphate in the normal dog has been studied. D-Glucose, L-Sorbose and D-Sorbitol produced a definite decrease, and D-Arabinose a clear cut rise. By statistical comparison with the variation encountered by taking successive blood samples with the same intervals and under the same experimental conditions, it seems that D-Galactose, L-Rhamnose and D-Xylose have some decreasing power. D-Fructose (pyranose) produced an increase at 45′. D-Arabinose showed inhibitory action on the decreasing effect of glucose and insulin. L-Arabinose exhibits some inhibitory action on the effect of glucose and at 15′ after glucose + insulin.

1. Wigglesworth, V. B., Woodrow, C. E., Smith, W., and Winter, L. B., *J. Physiol.*, 1922-23, v57, 447.

2. Harrop, G. H., and Benedict, E. M., PROC. SOC. EXP. BIOL. AND MED., 1922-23, v20, 430.

3. Perlzweig, W. A., Latham, E., and Keefer, C. S., *ibid.*, 1923-24, v21, 33.

4. Bolliger, A., and Hartman, F. W., *J. Biol. Chem.*, 1925, v64, 91.

5. De Venanzi, F., *Rev. Soc. Argent. Biol.*, 1949, v25, 120.

6. Owsiany, E., and Wierzuchowski, M., *Comp. Rend. Soc. Biol.*, 1930, v103, 416.

7. Wierzuchowski, M., Pieskow, W., and Owsiany, E., *Biochem. Z.*, 1931, v230, 146.

8. Levine, S., Loube, S. D., and Weisberg, H. F., *Am. J. Physiol.*, 1949, v159, 107.

9. Fiske, C. H., and Subbarow, Y., *J. Biol. Chem.*, 1925, v66, 375.

10. Nelson, N., *ibid.*, 1944, v153, 375.

11. Glaschenträger, B., and Lehnartz, E., *Physiologische Chemie,* Springer Verlag, Berlin, 1951.

Received July 18, 1957. P.S.E.B.M., 1957, v96.

Distribution of CO and Radiochromium in Blood and Tissues of Rabbit and Dog. II. Radiochromium.* (23530)

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In animal experiments designed to determine the localization of extravascular carbon monoxide (CO), excess of CO over radiochromium (Cr^{51}) in tissue homogenates as compared to their contents in blood was used as an index of localization. Cr^{51} in excess of CO was found in the lungs and spleen, which suggested that Cr^{51}-tagged red cells may be sequestered from the circulating blood in these organs(1). Because of the possibility that loss of tag from the blood might influence the results of blood volume determinations done with the Cr^{51} method, this phenomenon has been studied more fully.

Methods. The procedures used for tagging with Cr^{51}, *in vitro* handling of the blood, simultaneous administration of tagged cells and CO to the rabbits and dogs, and analysis of the blood and tissues have been described (1,2). In 10 dogs the sites of injection of the tagged cells and of sampling and the mode of death varied, as shown in Fig. 1. To test the effects of the *in vitro* handling of blood on the sequestration phenomenon, 25 rabbits were injected with cell suspensions that had been treated in various ways. Usually the cells were resuspended in saline after tagging and were stored overnight at 5°C, but in 12 experiments they were stored for 1 to 3 weeks. Half of these specimens were suspended in saline, the other half in their own plasma. Hemolysis was seen only in saline suspensions stored for 1 week or more. The cells in these preparations were washed until the supernatant appeared clear before they were injected into their respective donors. Otherwise the experiments were conducted as usual. The CO:Cr^{51} ratio of each tissue homogenate (R_t) was compared to the ratio in the animal's blood (R_b) to obtain an R_t:B_b ratio(1). When the R_t:R_b ratio of the tissue homogenate was less than one, it could be assumed that Cr^{51} had accumulated in that tissue(1).

Results. The results of the analyses of tissue homogenates and blood from the 10 dogs, expressed as R_t:R_b ratios, appear in Fig. 1. Ratios for spleen were less than one in all dogs killed by bleeding. The exsanguinated animals had small, pale spleens, which gave homogenates consisting mostly of gray pulp. No evidence of Cr^{51} accumulation in the spleen appeared in the dogs killed with sodium pentobarbital (Nembutal). Here, the spleens were large, filled with blood, and produced homogenates consisting mostly of blood. The lung ratios were also less than one in all but 2 experiments where the tagged cells had been injected into branches of the portal vein. Here, the livers showed an excess of Cr^{51}, while the lungs did not. Two animals were perfused with saline after bleeding. This accentuated the differences between tissue and blood ratios; the lowest values for spleen,

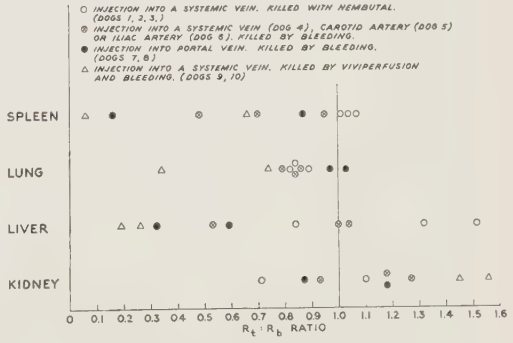

FIG. 1. $R_t : R_b$ ratios of tissue homogenates from dogs killed 20 minutes after receiving CO and Cr^{51}-tagged cells simultaneously. R_t = ratio in tissue homogenate of CO content (volume %) to Cr^{51} content (counts per second). R_b = ratio of CO to Cr^{51} content of the corresponding blood sample.

* This study was aided by grants from Edwards and Hertzstein Funds, allocated by Committee on Research, University of California School of Medicine, and from the U. S. Public Health Service and the San Francisco and Monterey County Heart Assns.

† Research Fellow, Am. Heart Assn., while this work was in progress.

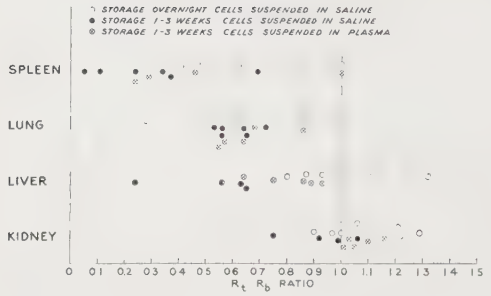

FIG. 2. $R_t : R_b$ ratios of tissue homogenates from 25 rabbits given autogenous red cells tagged with Cr^{51}, after storage for various intervals before injection, and CO. R_t and R_b are derived from tissue and blood analyses (see text and Fig. 1).

lung and liver were found in the experiment where the perfusion was particularly complete. After injection of the tagged cells into a common carotid artery the brain showed no retention of tag, but the usual excess was found in spleen and lung. When the injection was into the right external iliac artery, no excess of Cr^{51} was found in muscle or spongy bone of the right as compared to the left leg, and the usual retention occurred in lung and spleen. There was no evidence of chromium retention in the kidneys.

Fig. 2 shows the results of studies relating to the *in vitro* handling of the blood in 25 rabbits. The longer the storage time, the greater the excess of Cr^{51} found in lung, liver and spleen, particularly when the cells had been stored in saline.

An attempt was made to assay the total amounts of Cr^{51} lost from the cells after they had been handled and stored in various ways. Table I shows the amounts of Cr^{51} lost to the supernatant in experiments on blood taken from 8 rabbits and stored in saline or plasma for 1 day to 3 weeks. Table II shows the total amount of Cr^{51} retained in lungs, liver and spleen combined as % of the dose of Cr^{51} given to each of the 8 rabbits. The Cr^{51} content of the organs was determined by analysis of the entire organ (lung and spleen) or of duplicate aliquot samples (liver). The CO content of the homogenates and of the blood provided a means of correcting for the blood content of the organs.

Discussion. The exact mechanism responsible for sequestration of Cr^{51} is not shown by these studies, but must relate to physicochemical changes in the red cells caused by *in vitro* handling and to the functions of the reticuloendothelial system. Tompkins has described changes in erythrocytes when suspended in saline that are reversed by addition of plasma(3). Some of the injected erythrocytes were retained in the lung or liver, depending on which of these organs was traversed first; Cr^{51} accumulated in the spleen even after the cells had passed through the lung or liver. In contrast, no retention was found in the brain or extremities when their capillary beds were the first ones reached by the tagged cells. Sequestration of cells in the lung may be analogous to the trapping in this organ of leukocytes(4) or of macromolecules (5).

The more roughly the blood was handled, the greater the sequestration of Cr^{51}. With prolonged storage of tagged cells, more and more Cr^{51} was lost, particularly when the cells were resuspended in saline. However, even with 3 weeks of storage, the total loss of tag

TABLE I. Loss of Cr^{51} to the Supernatant after Storage of Tagged Rabbit Erythrocytes in Plasma or Saline at 5°C for 1 Day to 3 Weeks.

	Storage time	A Radioactivity of cell suspension	B Radioactivity of supernatant	B as % of A
		Counts/sec./ml		
Cells suspended in plasma	1 day	4,490	5.5	.1
	1 wk	2,743	5.5	.2
	2 "	2,380	7.0	.3
	3 "	1,905	11.2	.6
Cells suspended in saline	1 day	4,170	4.5	.1
	1 wk	5,420	37.0	.7
	2 "	2,130	21.4	1.0
	3 "	2,180	32.7	1.5

TABLE II. Combined Loss of Cr^{51} by Sequestration in Lung, Spleen and Liver of Rabbits Killed 20 Min. after Injection of Tagged Cells Stored in Plasma or Saline at 5°C for 1 Day to 3 Weeks.

	Storage time	A Radioactivity of inj. 10 ml of cell suspension (Counts/sec.)	B Radioactivity of total Cr^{51} retained in lungs, liver and spleen (Counts/sec.)	B as % of A
Cells suspended in plasma	1 day	33,750	35	.1
	1 wk	21,750	82	.3
	2 "	lost		
	3 "	14,500	232	1.6
Cells suspended in saline	1 day	30,000	55	.2
	1 wk	38,750	1,010	2.6
	2 "	16,500	471	2.8
	3 "	18,250	609	3.3

from the blood by leakage *in vitro* and by early sequestration was not more than 2 to 4% (Tables I and II).

Errors caused by *in vitro* leakage and sequestration would be inconsequential in the ordinary blood volume determination. If cells are stored only 1 day, even in saline, the combined loss amounts to 0.3% (Tables I and II), which would be equivalent to an error of 15 ml in the measurement of a 5,000 ml blood volume. Hence it seems unnecessary to add plasma to the suspending medium as recommended by Chaplin(6). Reported studies of red cell survival(2,7,8) have indicated that the losses of Cr^{51} which occur at longer intervals after injection of tagged cells are of much greater magnitude than were observed here (6% during the first 24 hours, then about 1% per day).

Summary. 1. Carbon monoxide and autogenous red cells labeled with Cr^{51} were administered simultaneously to dogs and rabbits; tissue homogenates and blood were then analyzed for radioactivity and CO content. 2. Small amounts of Cr^{51} accumulated regularly in spleen and lung and, when the cells were delivered via the portal vein, in the liver. No sequestration occurred in brain, kidneys or extremities. 3. Prolonged storage of tagged cells, particularly when suspended in saline rather than plasma, increased the loss of Cr^{51} from the cells *in vitro* and by early sequestration after injection of the cells into their donors. 4. When cells were stored in saline for only 1 day, combined losses caused by *in vitro* leakage and sequestration were too small to influence the results of blood volume determinations.

1. Wennesland, R., Nomof, N., Hopper, J., Jr., Brown, E., and Bradley, B., PROC. SOC. EXP. BIOL. AND MED., 1957, v96,

2. Nomof, N., Hopper, J., Jr., Brown, E., Scott, K., and Wennesland, R., *J. Clin. Invest.,* 1954, v33, 1382.

3. Tompkins, E. H., *J. Lab. and Clin. Med.,* 1954, v44, 554.

4. Bierman, H. R., Kelly, K. H., and Cordes, F. L., *Ann. New York Acad. Sc.,* 1956, v59, 850.

5. Waser, P., and Hunzinger, W., *Experientia,* 1951, v7, 6.

6. Chaplin, H., *J. Physiol.,* 1954, v123, 22.

7. Ebaugh, F. G., Jr., Emerson, C. P., and Ross, J. F., *J. Clin. Invest.,* 1953, v32, 1260.

8. Mollison, P. L., and Veall, N., *Brit. J. Haematol.,* 1955, v1, 62.

Received August 19, 1957. P.S.E.B.M., 1957, v96.

Occurrence of Intranuclear Inclusions in Tissue Cultures Infected with Virus of Infectious Bovine Rhinotracheitis. (23531)

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The disease now known as infectious bovine rhinotracheitis (IBR) appears to have first been recognized in 1950 in Colorado(1). Since that time it has been reported from a number of states in the western and midwestern part of the United States. Recent papers (2,3) have described the isolation and propagation of the etiological agent in tissue cultures of bovine cells. However, inclusion bodies have not been described in tissue culture or in naturally or experimentally infected animals(4).

It is the purpose of this report to describe the intranuclear inclusions which are a constant feature of the cytopathogenicity of this virus in cultures of bovine kidney and human amnion.

Materials and methods. Tissue cultures of bovine kidney were prepared by a modification of the trypsin digestion technic of Youngner(5). Cells were grown in tubes 16 x 150 mm incubated initially as stationary slant cultures at 35°C; after inoculation they were maintained as either stationary or roller cultures. The nutrient medium consisted of 95% lactalbumin hydrolysate-tris solution (0.5% lactalbumin hydrolysate in modified Hanks buffered salt solution containing 0.01 M tris [hydroxymethyl aminomethane] adjusted to pH of 7.4) and 5% lamb serum. Cultures of human amnion were prepared by the method of Zitcer, Fogh and Dunnebacke (6). Media consisted of 95% of either lactalbumin hydrolysate-tris solution or bovine amniotic fluid and 5% horse serum. Phenol red (0.002%), penicillin (100 U/ml), streptomycin (100 μ/ml) and mycostatin (100 U/ml) were incorporated in all media. Three strains of virus were studied. The first was provided through the courtesy of Dr. W. R. Pritchard, Purdue University, as fluid from the 15th tissue culture passage and is referred to as the Pritchard strain. The American and Cooper strains were obtained from Dr. T. L. Chow, Colorado A & M College, as nasal washings from clinical cases of IBR. Cytopathogenic agents were isolated and passed serially from both nasal washings in cultures of bovine kidney and the American strain in cultures of human amnion. Titration of virus was carried out using cells homologous for the tissue culture system being studied; calculation of ID_{50} was based on an inoculum per culture of 0.1 ml. After fixation in Bouin's fluid hematoxylin-and-eosin stained preparations were made employing either coverslips or the collodion membrane technic(7).

Results. With an inoculum of 100 ID_{50} of Pritchard strain of IBR lesions were first seen in cultures of bovine kidney at 18-24 hours but were generally somewhat more delayed in cultures of human amnion. In both tissues the lesions consisted of small foci of degeneration (Fig. 1). These foci slowly increased in size and new or secondary foci began to appear in an additional 24 hours in kidney and in 36-72 hours in human amnion. The degeneration generally became more diffuse in bovine kidney, involving the entire culture in about 72 hours, while in human amnion the focal nature of the change persisted longer, frequently requiring 10-14 days for total involvement of the cultures. The affected cells were rounded or spindle-shaped with elongated, occasionally beaded, processes. The cytoplasm was opaque and showed fine perinuclear granularity in contrast to the relative nuclear pallor. Nucleoli were small or inapparent. As the degeneration proceeded and foci became confluent the growth was converted into a network of these spindle-shaped cells which remained intact and adherent to the glass for many hours.

As indicated by the pH of the media there was increased acid production in the infected cultures.

A constant feature of the cytopathogenicity of all 3 strains studied was the occurrence in stained preparations of typical intranuclear inclusions in a large proportion of the degen-

erating cells (Fig. 2,3,4). These bodies began to appear at about the time degenerative changes were first detected in the unstained preparation. The earliest change in the nucleus usually consisted of a single small irregular aggregate of amphophilic to eosino-

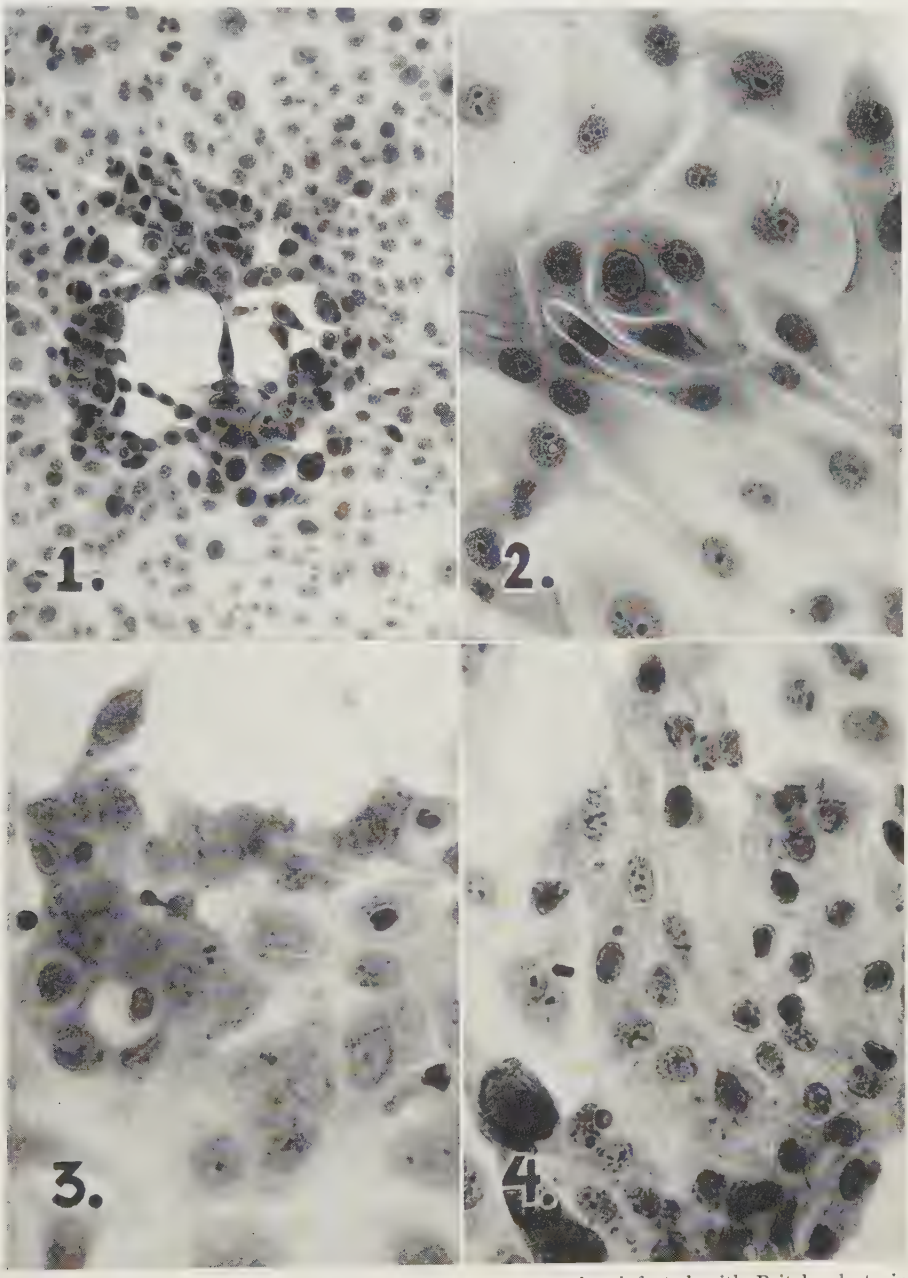

FIG. 1. Early lesion seen in tissue culture of human amnion infected with Pritchard strain of IBR. H & E × 130. AFIP neg. 57 2079.

FIG. 2. Intranuclear inclusion in tissue culture of human amnion infected with Pritchard strain of IBR. H & E × 265. AFIP neg. 57 2072.

FIG. 3. Intranuclear inclusions in culture of human amnion infected with Pritchard strain of IBR. H & E × 450. AFIP neg. 57 2080.

FIG. 4. Intranuclear inclusions in culture of bovine kidney infected with Pritchard strain of IBR. H & E × 450. AFIP neg. 57 4748.

philic, finely granular material among the nuclear chromatin. It appeared that the size of the particles responsible for the granularity of the inclusions was at about the limit of resolution of the light microscope, so that in some preparations the inclusions were homogeneous. The aggregate slowly increased in size until it occupied a major portion of the nucleus. By the time inclusions were well developed, nucleoli were generally small or had disappeared. These mature inclusions retained the amphophilic to eosinophilic staining of the earlier ones and were either round, oval or irregular in contour, generally corresponding to the shape of the nucleus. Although there was not the intense reaction of the nuclear chromatin characteristic of viruses of the herpes group, there was margination of the chromatin toward the nuclear membrane. Even in the late stages of degeneration the nuclear membrane usually remained intact and was separated from the inclusion by a thin clear zone or halo. Intense cytoplasmic eosinophilia accompanied the nuclear changes, but no specific cytoplasmic inclusions were seen. Changes similar to those observed with the Pritchard strain were also seen with the American and Cooper strains.

Cytopathogenicity, including the development of intranuclear inclusions, could be prevented by convalescent serum from clinical cases of rhinotracheitis. No evidence of neutralizing substances was found in the single pool of human gamma globulin tested.

Discussion. Previous reports of rhinotracheitis in tissue culture and of necropsy of naturally and experimentally infected animals have not described the presence of specific inclusions in this disease. In spite of these negative reports the demonstration of distinct intranuclear inclusions in tissue culture of three different strains of the virus of IBR, their consistent association with the cytopathogenicity and the prevention of their development by immune serum leave little doubt that they are a specific manifestation of cellular infection by this virus.

Although the metabolic activities responsible for the decreased pH of cultures inoculated with IBR have not been investigated, it is of interest that similar observations have been made in cultures of HeLa cells infected with adenovirus(8).

We have been able to demonstrate similar intranuclear inclusions in necropsy material fixed in Zenker's and Bouin's fluids. This material was obtained from calves killed during the early stages of disease following intranasal inoculation with the 3 strains of IBR. It appears likely that demonstration of such inclusions in nasal smears or biopsy specimens may facilitate diagnosis of this disease.

Summary. The virus of infectious bovine rhinotracheitis was isolated and passed serially in cultures of human amnion with the production of lesions similar to those occurring in cultures of bovine kidney. Specific intranuclear inclusions were noted in tissue culture and in cases of the disease experimentally produced with the 3 strains of virus studied.

1. Miller, N. J., *J. Am. Vet. Med. Assn.,* 1955, v126, 463.

2. Madin, S. H., York, C. J., and McKercher, D. G., *Science,* 1956, v124, 721.

3. York, C. J., Schwarz, A. J. F., and Estela, L. A., PROC. SOC. EXP. BIOL. AND MED., 1956, v94, 740.

4. McKercher, D. G., Moulton, S. E., Madin, S. H., and Kendrick, J. W., *Am. J. Vet. Res.,* 1957, v18, 246.

5. Youngner, J. S., PROC. SOC. EXP. BIOL. AND MED., 1954, v85, 202.

6. Zitcer, E. M., Fogh, J., and Dunnebacke, T. H., *Science,* 1955, v122, 30.

7. Enders, J. F., and Peebles, T. C., PROC. SOC. EXP. BIOL. AND MED., 1954, v86, 277.

8. Fisher, T. N., and Ginsberg, H., *ibid.,* 1957, v95, 47.

Received August 14, 1957. P.S.E.B.M., 1957, v96.

Serum Protein Changes Following Feeding of Parathion to Dogs. (23532)

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The use of paper electrophoresis for fractionation of blood serum as well as other body fluids has given the pharmacologist a valuable tool for evaluation of some toxic manifestations of new pharmacological agents. Alterations in serum protein fractions, for example, whether relative or absolute, have been shown to accompany many pathological states. Marrack and Hock(5) in their review of serum proteins indicate that disease processes which involved liver parenchyma resulted in increase in gamma globulin and decreased albumin.

The purpose of this study was to determine whether a subacute non-lethal level of parathion, (0,0-diethyl 0-*p*-nitrophenyl thiophosphate), would produce any electrophoretically observable changes in blood serum proteins of the dog. Simultaneously blood cholinesterase levels were also studied.

Materials and methods. Five control cholinesterase determinations were made on plasma and red cells on 2 male and 2 female dogs over a 4-week period, by the electrometric method of Michel(6) as modified by Frawley(7). These animals were then placed for 9 weeks on a diet containing 100 ppm. of parathion in Purina Laboratory Chow. Results of plasma and red cell cholinesterase determinations are plotted in Fig. 1 at the end of 5th, 6th, 7th, and 13th weeks. During this 9-week parathion feeding, 9 blood samples were drawn from the 4 parathion treated dogs and an equal number from 5 control animals. Ten ml blood samples were drawn by external jugular vein puncture, placed in 12 ml centrifuge tubes containing 0.25 ml of 1% lithium oxalate and centrifuged at 2730 rpm for 15 minutes in standard Adams safety head fixed angle (52°) centrifuge (12.7 cm radius). The unclotted plasma was then separated from the cells and caused to clot by addition of 0.25 ml of 1% calcium chloride solution. The clotted plasma was then centrifuged again for 15 minutes and the supernatant serum separated from clot. Ten μl of this serum were placed on the "Spinco" sample striper and spotted alternately with control samples on 8 "Spinco" Whatman No. 3 mm filter paper strips. The procedure from this point on is adapted from that described by Williams *et al.*(8) using the hanging-strip type cell. The electrophoretic run was either for 16 hours at 3 milliamperes or for 6½ hours at 20-22 milliamperes constant current at 25-31°C. Under these conditions the average albumin migration from point of origin was 8.2 cm. The barbital buffer was pH 8.6 with an ionic strength of 0.05. Upon completion of experiment the strips were removed from the cell and placed horizontally in a flat glass dish which was then placed in a 100-110°C oven for 45 minutes. The strips were then placed in a 0.01% acidified bromphenol blue solution for 16 hours. Three rinsing baths in 2% acetic acid were followed by 3 to 5 minutes in 10% acetic acid 2% sodium acetate fixer solution. Drying was on filter paper at room temperature. The strips were scanned in a "Spinco" Analytrol with automatic integrator.

Results. The results of 18 samples of blood serum drawn over 9 weeks of the experiment, on 5 control and 4 parathion fed dogs (2 male and 2 females each) on parathion and 2 males and 3 females on control are compiled in Table I.

The results indicate a statistically significant relative increase in the β-globulin component and decrease in the albumin fraction as a result of feeding of parathion.

The results of cholinesterase determinations

TABLE I.

		Globulins			
	Albumin	α_1	α_2	β	γ
Control	48.0	9.0	10.6	13.5	18.8
Parathion-fed (100 ppm.)	*34.15	7.9	11.6	*23.3	23.1

* Difference from control value is statistically significant.

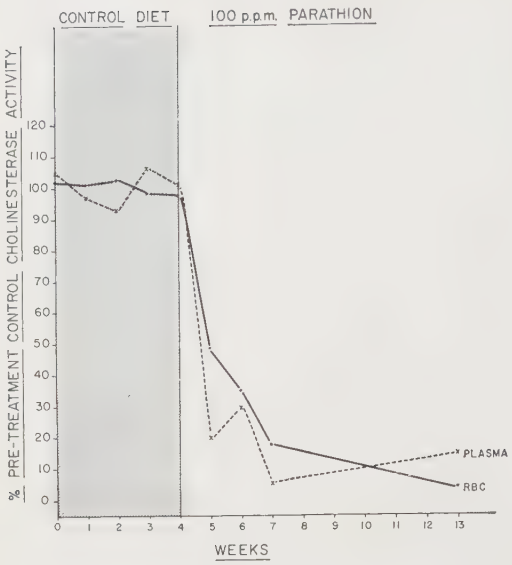

FIG. 1.

noted in Fig. 1 indicate the rapid decline in both plasma and red cell cholinesterase during the first 3 weeks of phosphate feeding. The terminal levels all below 20% of pre-treatment control indicate an injury which is also reflected in serum protein changes.

Fig. 2 shows typical serum sample patterns from both control and parathion fed dogs.

Discussion. As an index of toxicity, the study of serum protein changes offers promise. Marrack and Hock indicated(5) that the changes noted in serum proteins are not specific for any given pathological situation but yield an index to the overall constitutional state. The development of paper electrophoresis has provided the tool by which we may study the effects of toxic agents simply and rapidly without the necessity of elaborate and technically difficult older methods.

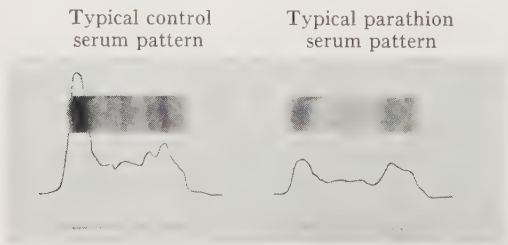

FIG. 2.

Much evidence to 1949(5) indicated that stressing agents, whether of functional, infectious or toxic nature, all produce rather typical changes in the blood serum picture. Whether we are dealing with syphilis, liver disease, malaria(1), liver ischemia(9), leg fractures(2), rickettsiosis, acute babesiosis (3) X-Ray(4) sulphur mustard, burns or turpentine(10), the resulting serum protein changes are essentially the same, namely, decreased albumin, both relatively and absolutely and a tendency for increased gamma and sometimes beta globulins. The present study further verifies that concept and points a new approach to the study of subacute functional damage from various toxic agents.

Summary. Serum electrophoretic patterns from dogs fed 100 ppm of organic phosphate parathion, indicate a relative decrease in level of albumin and increase in beta globulin. The significance of these findings is discussed.

1. Janssen, L. W., *Electrophoretic Studies on Serum Proteins,* North Holland Publishing Co., Amsterdam, 1951.

2. Cuthbertson, D. P., and Tompsett, S. L., *British J. Exp. Path.,* 1935, v16, 47.

3. Polson, A., and Malherbe, W. D., *Onderstepoort J. Vet. Res.,* 1952, v25, 13.

4. Muntz, J. A., Guzman-Barron, E. S., and Prosser, C. L., *Arch. Biochem.,* 1949, v23, 434.

5. Marrack, J. R., and Hock, H. J., *Clin. Path.,* 1949, v2, 161.

6. Michel, H. C., *J. Lab. and Clin. Med.,* 1949, v34, 1564.

7. Frawley, J. P., and Fuyat, H. N., *Agric. and Food Chem.,* 1957, v5, 346.

8. Williams, F. G., Pickles, E. G., and Durrum, E. L., *Science,* 1955, v121, 829.

9. Rappaport, A. M., Clarke, D. W., and Stewart, M., PROC. SOC. EXP. BIOL. AND MED., 1952, v80, 585.

10. Gjessing, E. C., Ludwig, S., and Chanutin, A., *J. Biol. Chem.,* 1947, v170, 551.

Received September 20, 1957. P.S.E.B.M., 1957, v96.

Concentration of Highly Potent Erythropoietic Activity from Urine of Anemic Patients.* (23533)

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Since the demonstration by Reissmann that erythropoiesis is under humoral control(1) the search for the site of production of this factor and for an adequate source from which erythropoietic hormone could be isolated has been the subject of much investigation. Erythropoietic activity has been found in a variety of body fluids, including plasma(2-4), urine (5,6), and milk(7), and erythropoietically active material has been isolated from the anterior pituitary(8). However, such activity has been demonstrated in body fluids primarily following some procedure which has decreased the oxygen-delivering ability of the blood (phenylhydrazine(2), hemorrhage(3), hypoxia(9), etc.), in disease states(4), or following administration of cobalt(10); from such conditions it has not been possible to isolate active fractions in sufficient quantity for adequate chemical and biological investigations.

In the course of screening a large number of patients with various hematological disorders for plasma erythropoietic titer, using the Fe^{59} incorporation in red cells as an assay (11,12), it was found that some patients with aplastic anemia showed a marked elevation in their titer. From the urine of some of these patients a highly potent erythropoietin has been extracted.

Material and methods. Normal female rats of the Long-Evans strain weighing between 150 and 200 g were used for the assay. They were fed a complete diet.† The rats were divided into groups of equal average weight at the beginning of any assay. Untreated urine or a concentrate of urine was injected subcutaneously once daily for 14 days. Control rats were injected with an equal volume of saline or urine from a normal subject. For comparison with the response obtained with urine concentrate, a group of rats was maintained continuously in a decompression chamber at a simulated altitude of 15,000 feet for 14 days. On the 12th day of injection, metabolic rates were determined with a closed-circuit multiple respiration apparatus, as described by Evans, *et al.*(13).‡ To measure I^{131} uptake by the thyroid, 0.5 μc of I^{131} was given intraperitoneally 16 hours prior to autopsy, and the activity of the thyroid determined after autopsy. Just prior to autopsy, blood volume and total circulating red cell volume were determined by the Fe^{59} labeled cell dilution method(14). At autopsy the adrenals, thyroid, ovaries, uterus, thymus, and spleen were dissected and weighed. The urine used in assays reported here was obtained from a Caucasian female of 11 years, who was first found to be anemic at the age of 1½ months and who has received transfusions monthly since the age of 3 months. Physical examination was essentially normal except for some enlargement of liver and spleen. Laboratory work has shown slightly hypochromic, normocytic erythrocytes, reticulocyte count of less than 1%, and only 2% nucleated red cells in the marrow with an essentially normal leukocyte formula. She has shown no response to therapeutic trials of cobalt, ACTH, riboflavin, and other conventional forms of therapy, such as liver, vit. B_{12}, etc. Iron turnover studies confirmed the other laboratory findings in that the marrow failed to accumulate and release radioiron for erythropoiesis. She has been diagnosed as erythroid hypoplasia. The urine concentrate

* This work was supported in part by the U. S. Atomic Energy Com.

† The diet (modified from McCollum's formula) consists of 67.5% whole wheat, 15% casein, 10% whole milk powder, 0.75% NaCl, 1.5% $CaCO_3$, 5.25% hydrogenated vegetable oil, and a concentrate of fish oil in amount to give 19 U.S.P. units of vit. A and 2.5 A.O.A.C. chick units of vit. D/g of diet.

‡ We wish to thank Dr. E. S. Evans for determinations of metabolic rate.

TABLE I. A Comparison of Hematologic Response of Normal Rats Injected with 1 ml of Untreated Urine Daily from a Normal and an Aplastic Anemia Donor.*

Material inj. 1 ml/day	Hct (%)	Red cell vol (ml/100 g body wt)	Total red cell vol (ml)	Increase in total red cell vol (%)
Saline control	45.6 ± 1.7†	2.39 ± .1	4.66 ± .2	
Normal urine	43.6 ± 2.6	2.32 ± .2	4.55 ± .2	0
Active "	49.9 ± 3.3	2.73 ± .2	5.34 ± .1	14.6

* 7 rats/group. † Mean and stand. dev.

was prepared by the ultrafiltration method described by Gorbman(15).

Results. A variety of normal human beings and patients with various hematological disorders were assayed for plasma erythropoietic activity by the Fe^{59} red cell incorporation assay using hypophysectomized rats. One ml of untreated plasma from normal human beings consistently gave an average Fe^{59} incorporation of 26%, as compared with 6% for the saline injected controls. No elevation in erythropoietic titer above normal was found in the patients assayed with the exception of 3 children with aplastic anemia, all of whom showed markedly elevated plasma erythropoietin, 1 ml of plasma producing a 40-50% incorporation of Fe^{59} in 16 hours. With such an elevation in plasma, it was decided to assay the urine. In 2 of the 3 children the erythropoietic activity of 1 ml of urine was as high as that of 1 ml of plasma, whereas normal urine shows no activity. In the third patient the urine showed no activity. If such an increase in iron incorporation after the administration of urine did in fact indicate a stimulation of erythropoiesis, then it should be possible to show an increase in the total red cell volume with a longer period of administration.

Table I compares the average values obtained in total circulating red cell volume with urine from the patient described above (1 ml daily for 14 days) with the values obtained with urine from a normal subject or with normal saline. The average hematocrit and red cell volume were significantly increased ($p = 0.001$, 15% above the saline-injected control) in the group injected with 1 ml of active urine. There was no change following injection of urine from a normal subject. Although the daily administration of only 1 ml of urine resulted in the production of a definite polycythemia in normal adult rats, it was felt that the true potency of this material could be demonstrated best by comparing the response obtained from larger amounts of urine with that obtained with the most effective known erythropoietic stimulant, hypoxia. For this purpose some method of concentration of the active principle from urine was sought. Since the ultrafiltration technic of Gorbman(15) provides a simple method for the concentration of gonadotrophic and possibly other pituitary hormones(16) in urine, the ultrafiltration of erythropoietically active urine was investigated. Under 20 lb nitrogen pressure, 500 ml of urine was forced through a collodion membrane. The membrane was dissolved in ether alcohol, and a precipitate was obtained by centrifugation. The precipitate was washed with ether, dried to a powder, and stored under vacuum. The powder was dissolved in saline, and an aliquot diluted to a volume equivalent to that of the starting material, while a second aliquot was dissolved so as to give a 13-fold concentration. The original urine, the ultrafiltrate, and the 2 dilutions of the residue remaining after ultrafiltration were assayed for erythropoietic activity by the Fe^{59} incorporation assay using normal rather than hypophysectomized rats. The use of normal rats in this assay has been

TABLE II. Partition of Erythropoietic Activity in Urine of a Patient with Aplastic Anemia.

Treatment	Vol equivalents inj. (ml)	Fe^{59} uptake* (16 hr) (%)
Starting material (untreated urine)	1	48 ± 6.3
Ultrafiltrate	1	28 ± 7.9
Residue after ultrafiltration	1	43 ± 7.6
Idem	13	61 ± 4.4
Uninjected control	—	23 ± 6.7

* Normal adult female rats; 10 rats/group.

TABLE III. Hematologic Values of Normal Rats Injected with Urinary Erythropoietic Fraction or Exposed to Simulated Altitude of 15,000 Ft for 14 Days.*

Treatment	Hct (%)	Red cell vol (ml/100 g body wt)	Total red cell vol (ml)	Increase in total red cell vol (%)
Saline	43.0 ± 1.66†	2.45 ± .1	4.48 ± .55	
Hypoxic control	49.9 ± 1.95	3.27 ± .2	5.76 ± .46	28.6
Urine fraction (2 mg/day)	56.8 ± 4.24	3.45 ± .3	6.70 ± .83	49.6

* 6 rats/group. † Mean and stand. dev.

found to be satisfactory when the activity of the material to be tested is sufficiently high. Table II summarizes the results of this assay. As can be seen from the table, no activity was found in the ultrafiltrate. The activity of the starting material and of the residue diluted to a volume equivalent to the starting material were equal. The more concentrated fraction showed a greater activity. All of the active material was recovered in the filter, providing a simple and efficient method for concentrating and purifying the activity and reducing it to a nontoxic, easily soluble powder. A urine fraction prepared in this way was injected subcutaneously into normal adult rats at a dose equivalent to 30 ml (2 mg) daily for 14 days. A second group was injected with saline and a third was maintained continuously in a decompression chamber at a simulated altitude of 15,000 feet for 14 days. On the 15th day total circulating red cell volumes were determined, and the animals were autopsied. Table III compares the average values for hematocrit and red cell volume for the 3 groups. As can be seen from the table, the total red cell volume of the rats maintained at a simulated altitude of 15,000 feet was 28.6% above that of the saline-injected controls, while those given an equivalent of 30 ml of active urine per day showed an increase in total red cell volume of 49.6% above the controls. Table IV compares average body weights, organ weights, and standard metabolic rates of the 3 groups. The rats injected with concentrate of active urine showed a greater increase in body weight than either the saline-injected or hypoxic controls, at least indicating that the injected material was not toxic. There was no significant change in the average weight of the endocrine organs or in the I^{131} uptake in the 3 groups. The spleens of the rats injected with urine concentrate showed a definite increase in weight, suggesting stimulation of erythropoiesis in that organ. The standard metabolic rate of the group injected with concentrate of active urine was only 9% above that of the saline-injected controls.

Thus, a certain group of patients classified as aplastic anemia produces and excretes erythropoietic substance in excessive quantities. It is suggested that in some cases the mechanism responsible for this high rate of production and excretion may be analogous to that responsible for the high level of follicle-stimulating hormone in the castrate or menopausal woman in that the production of the stimulant is uncontrolled because the target organ fails to respond, that is, there is no regulatory feedback mechanism. The source of production of the erythropoietic material found in plasma and urine has not been investigated, and one can only assume that that which is found in urine originates from that which is found in plasma.

TABLE IV. Organ and Body Weights and Metabolic Rates of Normal Adult Rats Injected with Urinary Erythropoietic Fraction or Exposed to Simulated Altitude of 15,000 Ft for 14 Days.*

Treatment	Body wt (g) Beginning	Body wt (g) Final	Adrenals (mg)	Thyroid (mg)	Ovaries (mg)	Uterus (mg)	Thymus (mg)	Spleen (mg)	Metabolic rate (cal/m²/hr)
Saline	171	183	59	12.5	67	388	245	845	31.5
Hypoxic control	171	177	51	13.1	65	304	192	890	
Urine fraction	171	194	59	13.0	67	281	211	1,305	34.4

* 6 rats/group.

Summary. 1) Some patients with aplastic anemia had exceptionally high levels of erythropoietic activity in plasma. The urine of these patients was assayed, and in some it was equally as potent as the plasma. As little as 1 ml daily for 14 days of untreated urine from such a patient produced a significant polycythemia in normal adult rats. When a concentrate of such urine, prepared by ultrafiltration, was injected at a dose equivalent to 30 ml daily for 14 days into normal adult rats, a polycythemia was produced which exceeded that resulting from exposure to a simulated altitude of 15,000 feet for the same period of time. 2) The high potency of such urine has allowed its assay in normal rats. This finding, together with the easy availability of urine and the efficiency of ultrafiltration as a method for preparing concentrates of urinary erythropoietic activity, should allow for a more rapid investigation of the chemistry and biology of this factor.

1. Reissmann, K. R., *Blood,* 1950, v5, 372.

2. Borsook, H., Graybiel, A., Keighley, G., and Windsor, E., *ibid.,* 1954, v9, 734.

3. Fried, W. L., Plzak, L., Jacobson, L. O., and Goldwasser, E., PROC. SOC. EXP. BIOL. AND MED., 1956, v92, 203.

4. Piliero, S. J., Medici, P. T., Pansky, B., Luhby, A. L., and Gordon, A. S., *ibid.,* 1956, v93, 302.

5. Hodgson, G., and Tohá, J., *Blood,* 1954, v9, 299.

6. Gurney, C. W., Goldwasser, E., Jacobson, L. O., and Pan, C., *J. Clin. Invest.,* 1957, v36, 896.

7. Grant, W. C., *Blood,* 1955, v10, 334.

8. Van Dyke, D. C., Simpson, M .E., Contopoulos, A. N., and Evans, H. M., *ibid.,* 1957, v12, 539.

9. Prentice, T. C., and Mirand, E. A., PROC. SOC. EXP. BIOL. AND MED., 1957, v92, 231.

10. Goldwasser, E., Jacobson, L. O., Fried, W., and Plzak, L., *Science,* 1957, v125, 1085.

11. Hennessy, T. G., and Huff, R. L., PROC. SOC. EXP. BIOL. AND MED., 1950, v73, 436.

12. Plzak, L. F., Fried, W. L., Jacobson, L. O., and Bethard, W. F., *J. Lab. and Clin. Med.,* 1955, v46, 671.

13. Evans, E. S., Contopoulos, A. N., and Simpson, M. E., *Endocrinology,* 1957, v60, 403.

14. Berlin, N. I., Huff, R. L., Van Dyke, D. C., and Hennessy, T. G., PROC. SOC. EXP. BIOL. AND MED., 1949, v71, 176.

15. Gorbman, A., *Endocrinology,* 1945, v37, 177.

16. Greenspan, F. S., Moses, L. E., Kriss, J. P., and Lew, W., *Clin. Res. Proc.,* 1956, v4, 60.

Received September 10, 1957. P.S.E.B.M., 1957, v96.

Effect of Corticoids upon Skeletal and Renal Changes Produced by Stylomycin Aminonucleoside.* (23534)

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The literature on stylomycin aminonucleoside (SAN) is somewhat confusing, owing to the fact that the antibiotic now known as "stylomycin" was first called "achromycin"—a name which was subsequently transferred to another antibiotic(1)—and then "puromycin"(2,3,4). Little is known as yet about the pharmacology of this antibiotic, apart from its efficacy in the treatment of infections, particularly those caused by certain trypanosomes. It has been noted, however, that stylomycin and its aminonucleoside can produce a nephrosis-like syndrome with renal lesions, subcutaneous edema, pleural fluid, ascites, hyperproteinemia, hyperlipemia and azotemia in laboratory animals(1,3). This condition is accompanied by an increased aldosterone secretion(4), osteitis-fibrosa-like changes in the bones(5) and, in advanced intoxication, by an interstitial pancreatitis(6).

The object of the present communication is to report upon experiments which show that both the renal and the skeletal changes elicited by SAN overdosage depend largely upon the balance between pro- and anti-inflammatory corticoids.

* Supported by National Research Council of Canada and by Lederle Laboratories Division of Amer. Cyanamid Co., the latter having also supplied the stylomycin aminonucleoside used.

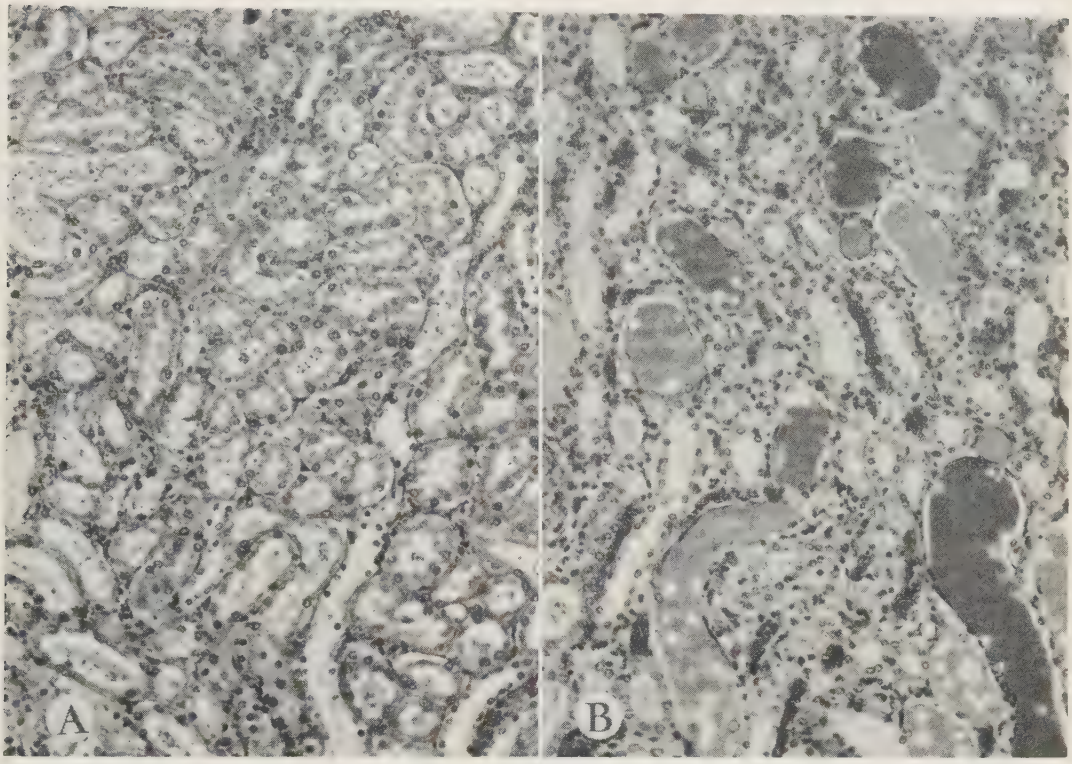

FIG. 1. Renal changes following combined treatment with SAN and corticoids. A. Virtually normal renal cortex following treatment with SAN + COL-Ac in a rat of Group II (X 150). B. Numerous hyalin casts and more or less granular protein precipitates in renal cortex of a rat of Group IV, treated with SAN + DOC-Ac (× 150).

Materials and methods. Fifty female Sprague-Dawley rats, with an average initial body-weight of 106 g (range: 98-112 g), were subdivided into five equal groups, all of which received 1 mg of SAN* in 0.2 ml of water subcutaneously, daily. *Group I* was not otherwise treated. The other animals received, in addition to the SAN, injections of corticoids as follows: *Group II*: cortisol acetate (COL-Ac)† 1 mg of microcrystals in 0.2 ml of water subcutaneously, once daily; *Group III* was adrenalectomized and treated with COL-Ac, as Group II; *Group IV*: desoxycorticosterone acetate (DOC-Ac)† 3 mg of microcrystals in 0.2 ml of water subcutaneously, once daily; *Group V* was adrenalectomized and treated with DOC-Ac, as Group IV. After 17 days the animals were killed with chloroform. The femurs, ribs and kidneys were fixed in Susa, embedded in paraffin and stained with hematoxylin-eosin, for subsequent histologic study. The acidity of the Susa solution sufficed to decalcify the skeletal structures within two or three days.

Results. It was evident at autopsy that the ascites and pleural fluid accumulations characteristic of SAN overdosage were most pronounced in the rats which received DOC-Ac (Groups IV and V), but there was no significant difference in this respect between the rats treated with SAN alone (Group I) and those which, in addition, were also given COL-Ac (Groups II and III).

On the other hand, histologic examination revealed that the renal changes, which were of moderate intensity in the controls, were diminished by COL-Ac (Groups II and III), and greatly aggravated by DOC-Ac (Groups IV and V). In Groups IV and V, the convoluted tubules were replete with hyalin casts and, occasionally, fibrinoid material appeared in the parietal laminae of Bowman's capsules

† Generously supplied by Schering Corp.

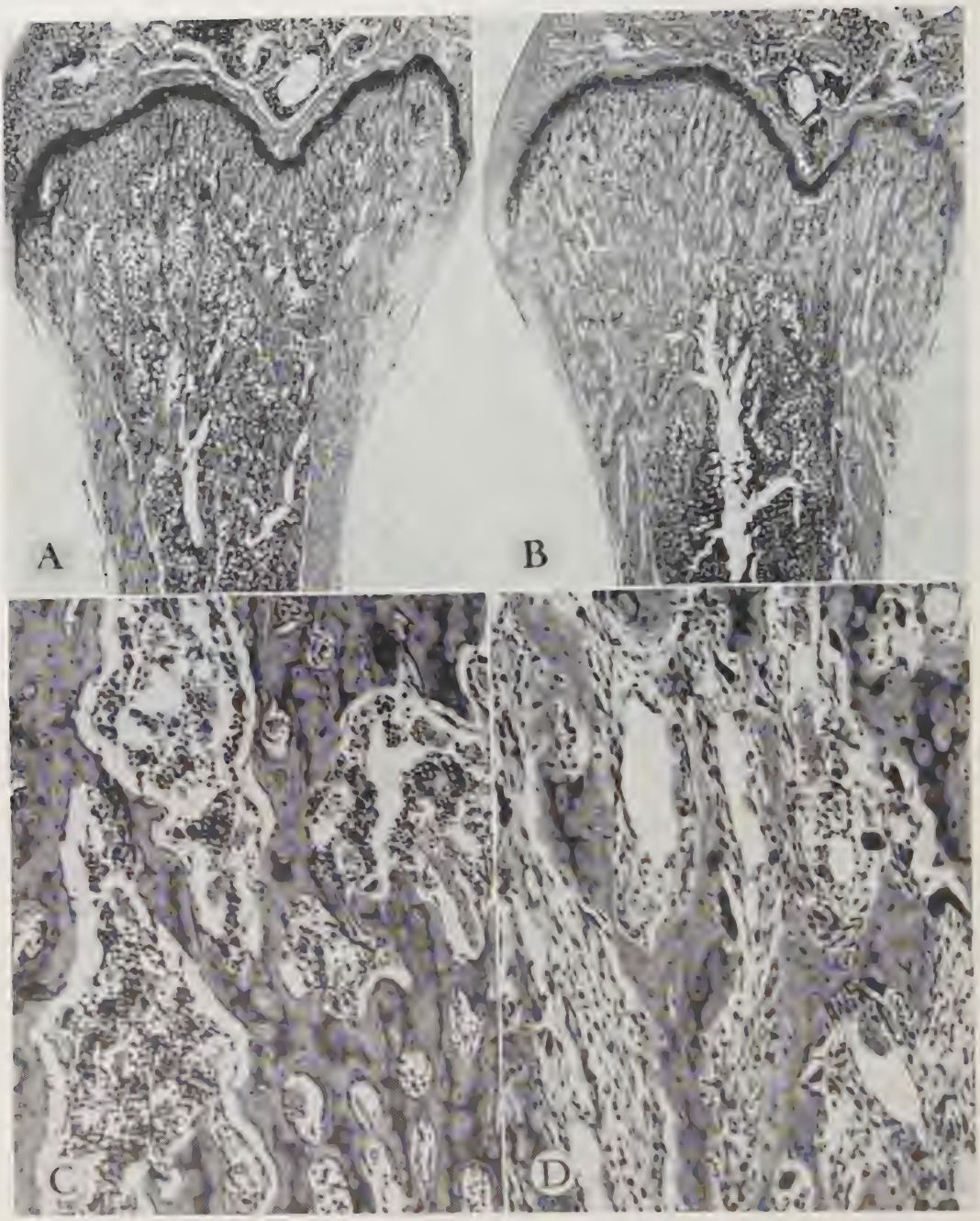

FIG. 2. Skeletal changes following combined treatment with SAN and corticoids. A. Cross-section through distal end of femur of a rat of Group II, which was treated with SAN + COL-Ac. Skeletal structure is essentially normal (X 15). B. Distal end of femur of a rat of Group IV, treated with SAN + DOC-Ac. Here the myeloid tissue has almost completely disappeared and is replaced by light, fibrous tissue between the trabeculae underneath the growth cartilage (X 15). C. Higher magnification of a region from Fig. A. Bone structure essentially normal (X 120). D. Higher magnification of a region from Fig. B. Here the myeloid elements are replaced by fibrous tissue with numerous, large (dark) osteoclasts (× 120).

or free in the glomerular spaces. It is known from previous work that, under similar conditions (that is, in short-term experiments and without "conditioning" by unilateral nephrectomy plus an excess NaCl), DOC-Ac produces no detectable signs of renal damage(7).

This has again been verified on 2 groups of 10 rats each, treated as the Groups IV and V of the previous experiment, but not given SAN.

On the other hand, chronic overdosage with DOC-Ac in suitably "conditioned" rats causes malignant nephrosclerosis with hyalinization of the afferent glomerular arterioles(7), that is, a change qualitatively different from that observed here (Fig. 1).

The femurs and ribs exhibited manifestations of mild osteitis fibrosa in Group I. The bone-marrow elements were partly replaced by a fibrous tissue that contained many osteoclasts. These changes were almost completely suppressed in the two groups that received COL-Ac (Groups II and III) and enormously aggravated in the animals treated with DOC-Ac (Groups IV and V). There was no significant difference, however, between the intact and the adrenalectomized animals, either as regards the suppression of renal and skeletal changes by COL-Ac, or their aggravation by DOC-Ac (Fig. 2).

Discussion. It is not known whether the osteitis fibrosa produced by SAN represents a form of "renal rickets." Parathyroidectomy, which prevents the development of osteitis fibrosa after nephrectomy(8), does not abolish the histologically similar lesions induced by SAN(5). This suggests that SAN can influence the bones through a mechanism other than an increased parathyroid hormone secretion. The experiments reported here furnish no information concerning the possible interrelations between the renal and skeletal manifestations of SAN intoxication, since COL-Ac inhibited, while DOC-Ac exacerbated both; hence it is impossible to say whether the action of the corticoids upon the bones was a consequence of their effects upon the kidney. It is also evident that SAN does not affect the renal or skeletal tissues merely by virtue of changes in corticoid secretion, since adrenalectomy does not prevent its toxic actions upon these structures. However, these observations furnish yet another instance of morbid changes, whose development is decisively influenced by the "conditioning" influence of the balance between pro- and antiphlogistic corticoids.

Summary. Experiments on albino rats indicate that stylomycin aminonucleoside (SAN) produces renal changes, accompanied by manifestations of osteitis fibrosa. Both renal and skeletal lesions elicited by SAN are inhibited by cortisol acetate (COL-Ac) and aggravated by desoxycorticosterone acetate (DOC-Ac).

1. Hewitt, R. I., Wallace, W. S., Gumble, A. R., Gill, E. R., and Williams, J. H., *Am. J. Trop. Med. and Hyg.*, 1953, v2, 254.

2. Baker, B. R., Schaub, R. E., Joseph, J. P., and Williams, J. H., *J. Am. Chem. Soc.*, 1954, v76, 4044.

3. Frenk, S., Antonowicz, J., Craig, J. M., and Metcoff, J., PROC. SOC. EXP. BIOL. AND MED., 1955, v89, 424.

4. Singer, Bertha, *Endocrinology*, 1957, v60, 420.

5. Selye, H., *ibid.*,

6. ———, *Am. J. Gastroenterol.*,

7. ———, *Stress*, 1950, Acta, Montreal.

8. ———, *Arch. Path.*, 1942, v34, 625.

Received August 19, 1957. P.S.E.B.M., 1957, v96.

Inhibition of Walker Tumor by Autolyzed Lymphosarcoma Cells.* (23535)

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The concomitant growth of 2 different tumors transplanted in the same host has been observed either to inhibit or to promote the development of the involved tumors. Bruwer *et al.*(1) were the first to report that a growing Murphy Rat Lymphosarcoma (MRLS) could inhibit the growth of a Walker carcinoma transplanted in the same site. We later observed the same phenomenon and wanted to verify whether the inhibition was due merely to competition of the two growing tumors for an essential factor or whether the mechanism was more likely to be a direct action of the lymphomatous cells on the Walker neoplasm.

To study the effect of non-viable lymphosarcoma, we used a 3-day-old suspension of MRLS cells in saline, for we knew that this partly autolyzed suspension does not take when injected. The Walker tumor was transplanted in an air-pouch as this method is ideal for testing local actions.

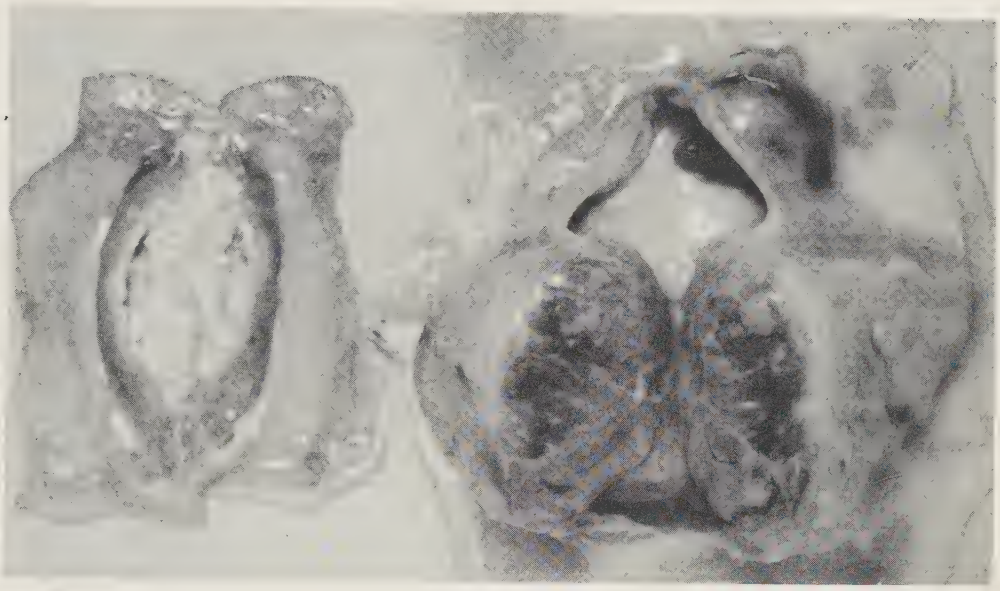
Materials and methods. Sixty-five female Sprague-Dawley rats of the same age, weighing approximately 150-170 g, were distributed into groups each having a mean bodyweight of 160 g. The animals were fed exclusively Purina Fox Chow and received tap water to drink. The Walker neoplasm was transplanted by injecting 0.5 ml of a suspension containing 0.2 g of crushed tumor tissue per ml of a 1% saline solution. The autolyzed suspension of MRLS is prepared in the same way, but left 3 days in a closed, sterile container, at room temperature, before it is injected. An air-pouch is made by injecting 25 ml of air into the subcutaneous tissue situated between the shoulder blades of a rat. When a tumor suspension is introduced into the cavity of the air-bubble thus formed, a tumor-pouch is produced(2). Our present study consisted of 3 experiments. In the first, we used 25 rats divided into 2 groups. All animals received a Walker tumor transplant in an air-pouch. Group 1, composed of 10 rats, was given no other treatment and served as control. Group 2 consisted of 15 rats which received, immediately after the tumor suspension, an injection of 0.5 ml of autolyzed MRLS in the air-pouch. Our second experiment was an exact repetition of the first, except that two groups of 10 rats each were used. In the third experiment, 2 groups of 10 rats each were again used. An air-pouch was made on the back of all the animals. The experimental group received 1 ml of autolyzed MRLS in the cavity of the pouch, while the controls had no injection. After 10 days, both groups were given 1.5 ml of Walker tumor suspension. The air-pouches in the control group had to be partly reinsufflated to make them equal in volume to the MRLS injected pouches.

Results. In the first of the experiments with concomitant injection of Walker carcinoma and autolyzed MRLS, none of the treated animals showed any apparent tumor development on the 17th day after transplantations while all the control rats had died, bearing large Walker tumors. One of the treated animals was then killed and the site of transplantation compared with that of the last control to die (Fig. 1). Only necrotic tissue could be found in the MRLS treated rat. On the 24th day, tumor nodules began to appear at the periphery of the pouch in 4 of the remaining 14 rats. The tumor grew to a large size around the MRLS injected area and killed the 4 animals on the 43rd and 44th days. The remaining 10 rats were then killed and autopsied. No tumor could be found. The pouch had perforated and the necrotic tissue had been eliminated. Only scar tissue remained in the inter-scapular region.

Repetition of the same experiment gave the following results: Of the 10 controls, all de-

* This work was supported by grants from the National Cancer Inst. of Canada and the Gustavus & Louise Pfeiffer Research Fn.

† Fellow of the National Cancer Institute of Canada, 1956.



(a) (b)

FIG. 1. (a) Necrotic tissue in a 17-day-old pouch injected with both Walker tumor and autolyzed MRLS. The bearer was killed for photographic purposes. (b) Pouch of the same age containing Walker tumor alone. The bearer was the last of its group to die.

veloped large tumors and died within 15 days. None of the MRLS treated animals showed any sign of tumor until the 20th day, when nodules began to be visible around the pouch. These tumors developed and finally killed the bearers.

In the third experiment, the Walker tumor injected in the pouch prepared with autolyzed MRLS did not grow and was eliminated through perforations in the outer wall of the pouch in 7 of the 10 animals. In the remaining 3, late tumor nodules started to grow 3 weeks after transplantation, while all controls had died with large tumors before the end of the 2nd week.

Conclusions. 1. The inhibition of Walker tumors by MRLS tissue does not depend upon concomitant growth of the 2 tumors. Autolyzed, non-growing MRLS is also effective in preventing or retarding Walker tumor development. It seems that, when the inflammation caused in the air-pouch by the autolyzed suspension is sufficient to perforate the wall of the pouch and eliminate its content, no tumor will later develop. On the other hand, if no perforation occurs, the carcinoma eventually grows but is considerably retarded. The animals injected with MRLS survive sometimes as much as 2½ times (experiment 1: 43/17 days) as long as the untreated tumor bearers. Undoubtedly this is not the result of any type of inflammation, for we have studied the action on tumor growth of many irritants and autolyzed tissues, and none had an effect comparable to that of autolyzed MRLS(3). 2. The termination of the inhibitory phase seems to depend on the age of the Walker transplant (about 3 weeks) and not on the duration of the inflammation caused by autolyzed MRLS. Indeed, the tumor development is prevented for the same length of time, either by a 10-day-old MRLS-pouch or by a newly formed MRLS inflammation.

Summary. Autolyzed, non-viable lymphosarcoma tissue, when injected, either before or simultaneously, in the same site as Walker tumor, can inhibit or retard the growth of the latter.

1. Bruwer, A., Donald, T. C., Jr., Higgins, G. M., McDonald, J. R., and Leddy, E. T., *Cancer Research,* 1951, v11, 922.

2. Selye, H., and Horava, A., 3rd Annual Rep. on Stress, 1953, 412, Acta Inc., Med. Publ., Montreal.

3. Richer, C. L., Thesis, 1957, Université Montréal.

Received September 20, 1957. P.S.E.B.M., 1957, v96.

Conversion of Pleuropneumonialike Organisms to Bacteria.* (23536)

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The relationship of pleuropneumonialike organisms (PPLO) to L forms and to bacteria is unknown. By definition the difference between L forms and PPLO is that the L forms are known to be associated with some bacterial species while the PPLO are not. Although there are innumerable instances of the production of L forms from bacteria and the reversion of the L forms to bacteria, no definitive proof of such a conversion of PPLO to a bacterium has been reported. Minck(1) reported that 5 PPLO isolates from the female genital tract developed into diphtheroids when inoculated into broth. McKay and Taylor(2) described the reversion of poultry strains of PPLO to bacteria similar to *Hemophilus gallinarum* when inoculated from fowl or chick embryo onto solid medium in the presence of added carbon dioxide and partial vacuum. They concluded that these organisms were not PPLO but L forms. Peoples *et al.*(3) reported on the association of *Corynebacterium* and the Campo strain of PPLO. Recently Wittler *et al.*(4) described the reversion of a strain of PPLO isolated from the human urethra to *Corynebacterium* following the inoculation of tissue cultures. This *Corynebacterium* was indistinguishable from another *Corynebacterium* isolated from the same urethral specimen.

A crucial test to establish the relation of PPLO to bacteria consists of causing the conversion of a well established strain of PPLO to a bacterium, reliably establishing the relationship of the two, and finally bringing about the production of the strain of PPLO from the derived bacterium. This report relates the findings of such an experimental attempt.

Materials and methods. The strain of PPLO employed for most of this study was Campo "L", obtained in 1949 from Dr. Dienes who isolated it from the human urethra and characterized it as a typical PPLO. It has been maintained on artificial solid medium for more than 10 years without evidence of reversion to a bacterium. In our laboratory it has been subcultured weekly on solid medium for several years. Two other strains, V73, isolated in our laboratory from the human urethra(5), and SF29, isolated by Carter(6) from the lung of a calf with bronchopneumonia, were examined with respect to association with bacteria. Statistical tests were designed and calculations performed by Dr. Max Woodbury, New York University. The tests involved were of the chi square method modified to account for (1) the probability that contamination of cultures occurred, (2) the probability that the contaminant could be a diphtheroid and (3) the probability that the contaminant could be a nondiphtheroid. Antigens for PPLO were prepared by growing the organisms in large quantities as previously described(7), with the exception that penicillin was added at a level of 100 u/ml to prevent the formation of diphtheroids. Antigens for the diphtheroids were prepared by harvesting 18-24 hour cultures grown in heart infusion broth. Both types of organisms were sedimented under sterile conditions in a Servall angle centrifuge, repeatedly washed in saline and resuspended in saline with "Falba" added as adjuvant. Considerably better titers of antisera were obtained with the use of the adjuvant. Duplicate albino rabbits were injected intramuscularly with 2 ml of the "Falba" adjuvant antigen, weekly, until each animal had received approximately 2.0 mg of cellular nitrogen. The animals were rested for one week and bled by cardiac puncture. Antigens for agglutination tests consisted of saline suspensions of organisms adjusted to give a Klett reading of 100. Standard tube agglutination tests were found to be satisfactory and therefore were used for all serological tests. Fermentation tests were run in either bromcresol purple or phenol red broth with added one per cent carbohydrate. Tubes were ob-

* This work was supported in part by contract between Office of Naval Research and the Univ. of Pennsylvania.

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served for acid production and growth over a period of 2 weeks.

Results. Considerable variation in the growth of the Campo strain of PPLO has been noted over the past 5 years. In addition to loss of the requirement for the lipoprotein growth factor(8), it has been observed that when large volume broth cultures of this strain were grown for use in metabolic studies bacteria were encountered very frequently in the absence of thallium acetate. The bacteria never were observed to appear on agar cultures of the Campo strain of PPLO and rarely in small numbers of 10 ml broth cultures. The high frequency of these bacteria in large volume cultures prompted an investigation of the incriminating organisms. Upon subculture onto solid medium, the bacteria grew as very minute colonies, approximately 5x the diameter of the Campo colonies. They were slightly raised with irregularly shaped, dense centers, could be transferred with an inoculating needle and grew without the added PPLO serum fraction. However, added 5% yeast extract greatly stimulated growth. Morphologically, they were Gram-positive, short, club-shaped rods and occasionally beaded. Fermentation tests revealed that like the Campo strain of PPLO, they were inert to all the carbohydrates tested. On the basis of these findings, the bacterium was identified as belonging to the genus *Corynebacterium*. Similar results were obtained with strains V73 and SF29.

Statistical tests. Initially, attempts were made to establish on a statistical basis whether the diphtheroids were appearing as contaminants or whether a relationship existed between them and the PPLO strain. Consequently, 2 statistical tests were devised. In the first experiment, 100 tubes, each containing 10 ml of the PPLO medium, were inoculated with agar blocks containing colonies of the Campo strain. These agar blocks did not contain any bacterial colonies upon observation at 100X magnification. An additional like 100 tubes were inoculated with sterile agar blocks. The investigator performing the inoculation was unaware of the type of agar block used for inoculum. Following 3 through 7 days of incubation at 37°C all the tubes were subcultured onto solid medium and those appearing to contain bacteria were examined by Gram staining. PPLO was found to be growing in all tubes inoculated with the Campo strain. Table I shows the results of this first experiment. Statistical analyses revealed that the probability of the diphtheroids being a contaminant was less than 1 in 10,000.

TABLE I. Frequency and Type of Isolations after Inoculation of 100 Tubes of Broth.

	1st exp.		2nd exp.	
	Inoc. with Campo PPLO	Control, inoc. with sterile agar blocks	Inoc. with Campo PPLO	Control, inoc. with sterile broth
Campo only	79		93	
Sterile		96		98
Diphtheroids	18	0	5	1
G-positive cocci	1	0	2	0
G-negative rods	2	4	0	1
Total	100	100	100	100
X^2	20.162		2.886	
P	1:10,000		1:10	

Since there was a chance that microscopic bacterial colonies were present on the agar inoculum and since the diphtheroids appeared only in liquid medium, a test was devised to select against the occurrence of the bacteria. The second experiment was identical to the first except that the inoculum used was 0.1 ml of pooled broth cultures of the Campo strain free from bacterial growth and of sterile broth. Broth cultures of the Campo strain prepared for inoculum which exhibited diphtheroids were discarded. The results of this second experiment are shown in Table I. As would be expected when employing an inoculum selected against the formation of diphtheroids, the probability that the diphtheroids were contaminants increased. However, it was still within the limits of 10%.

Repeated experiments of these 2 types by different investigators gave approximately the same results over a period of 3 years. However, it was found that the addition of 5% yeast extract aided in the production of the diphtheroids.

TABLE II. Cross Agglutination between PPLO Strains Expressed as Reciprocal Titers.

Antisera	Antigen: Campo PPLO	O7 PPLO	J PPLO
Campo PPLO	1024	32	1024
O7 "	"	1024	"
J "	0	0	2048

Biochemical tests. Examination of the fermentative ability of 15 diphtheroids isolated from Campo PPLO cultures and the Campo strain of PPLO showed that neither of these 2 types of organisms produced acid in the presence of glucose, maltose, sucrose, lactose, fructose, mannitol, inositol and salicin. The organisms multiplied in these test cultures. All diphtheroids isolated as air contaminants and from the oral cavity of the investigators were shown to be capable of producing acid from some of the above mentioned carbohydrates.

Serological tests. Although statistical tests indicated that the diphtheroids were not contaminants and that the diphtheroids isolated from Campo cultures differed from diphtheroids isolated from the air and the oral cavity in their fermentative capacity toward carbohydrates, a more reliable relationship was sought by the use of serological tests.

Table II shows that a relationship exists between 2 human urethral strains, Campo and 07, and a poultry strain, J, of the pleuropneumonialike group. The inability of the J antiserum to agglutinate Campo and 07 antigens can be explained by interference of antibody production to a minor antigen by a major antigen(9).

The cross agglutination reactions of the Campo PPLO, the Campo diphtheroid (Campo D) and the V73 diphtheroid (V73D) are shown in Table III. PPLO strains, 07 and J, failed to react significantly with the diphtheroids, although there is a cross reaction between these 2 PPLO and the Campo PPLO. No antigen or antiserum was prepared for the V73 PPLO because of the poor capacity of this strain to grow in liquid medium. Table IV shows the results of cross agglutination reactions between the Campo PPLO, the Campo diphtheroid and the V73 diphtheroid after reciprocal adsorptions. As can be seen reciprocal adsorptions removed the common antibodies in all instances.

TABLE III. Cross Agglutination between PPLO and Diphtheroids.

Antisera	Antigen: Campo PPLO	Campo D	V73 D
Campo PPLO	1024	64	16
" D	16	1024	128
V73 D	512	256	1024
PPLO strain, O7	1024	ND	0
" " , J	0	"	4

ND = not done.

Conversion of diphtheroids to PPLO. Numerous attempts have been made to derive a PPLO from the diphtheroids isolated from Campo cultures. In no instance was an L type colony reliably identified. Among the methods used in these attempts were the use of high concentrations of penicillin with and without added salts(10), cold shock and the use of glycine(11).

TABLE IV. Cross Agglutination after Adsorption with PPLO and Diphtheroids.

Antisera	Cells used for adsorption	Antigen: Campo PPLO	Campo D	V73 D
Campo, PPLO	Unadsorbed	1024	64	16
	Campo, PPLO	0	0	0
	" D	64	0	ND
	V73 D	64	ND	0
Campo D	Unabsorbed	16	1024	128
	Campo, PPLO	0	512	ND
	" D	0	0	32
	V73 D	ND	8	0
V73 D	Unadsorbed	512	256	1024
	Campo, PPLO	0	ND	16
	" D	ND	0	4
	V73 D	0	16	0

ND = not done.

Discussion. The combined results of the various studies established that a relationship exists between the diphtheroids isolated from the PPLO cultures and certain strains of PPLO. First, statistically designed tests showed that the bacterium found most frequently in the PPLO cultures, a species of *Corynebacterium,* was not a contaminant, even when the inoculum was selected against the presence of PPLO capable of producing diphtheroids. Second, morphological and biochemical studies indicated that the diphtheroids isolated from the PPLO cultures were identical with one another but different from air and oral diphtheroids. Third, a definite serological re-

lationship was found between those PPLO giving rise to diphtheroids and the derived diphtheroids but no serological cross reaction between PPLO strains not giving rise to diphtheroids and the derived diphtheroids. Although it is apparent that the Campo and V73 strains can be converted to diphtheroids, final confirmation of the phenomenon cannot be made until serological tests show the diphtheroids from PPLO cultures are similar or identical with one another but different from contaminant diphtheroids and until the diphtheroids are converted to a PPLO similar or identical to the parent PPLO.

Eventual establishment of a relationship between all PPLO strains and some microbial species is not improbable. The conversion of bacteria to a stable L form can be considered to involve a mutation or mutations, either spontaneous or induced. Conversion of a PPLO, which can be considered a stable L form, although the related bacterium is unknown, to a bacterium can likewise be considered to involve a mutation or mutations. In the case of the 3 strains which exhibited conversion to diphtheroids in this study, spontaneous mutation could be operative. Other strains, which have not shown a tendency to convert may have such a low mutation rate that conversion is not evident. If the proper conditions were employed conversion might occur in all PPLO strains. Presently these conditions are unknown. Additional evidence that PPLO may be related to some microbial species is the widespread ability of bacteria to form L colonies and protoplasts.

Summary. A high frequency of appearance of diphtheroids in liquid culture of 3 established strains of pleuropneumonialike organisms has been noted. Statistical, morphological, biochemical and serological tests have shown that a relationship exists between these diphtheroids and the pleuropneumonialike organisms in which cultures the bacteria appear. All PPLO strains tested are serologically related. PPLO strains not giving rise to diphtheroids do not cross react with diphtheroids. Diphtheroids appearing in PPLO cultures differ from oral and air diphtheroids.

1. Minck, R., *Compt. rend. acad. sci.,* 1953, v236, 250.

2. McKay, K. A., and Taylor, J. R. E., *Canad. J. Comp. Med.,* 1954, v18, 7.

3. Peoples, D. M., Smith, P. F., and Morton, H. E., *Bact. Proc.,* 1955, 49.

4. Wittler, R. G., Cary, S. G., and Lindberg, R. B., *J. Gen. Microbiol.,* 1956, v14, 763.

5. Peoples, D. M., Morton, H. E., and Feo, L. G., *J. Bact.,* 1957, v73, 398.

6. Carter, G. R., *Science,* 1954, v120, 113.

7. Smith, P. F., *J. Bact.,* 1955, v70, 552.

8. Smith, P. F., Lecce, J. G., and Lynn, R. J., *ibid.,* 1954, v68, 627.

9. Raffel, S., *Immunity, Hypersensitivity, Serology,* Appleton-Century-Crofts, N. Y., 1953, 69.

10. Sharp, J. T., PROC. SOC. EXP. BIOL. AND MED., 1954, v87, 94.

11. Dienes, L., Weinberg, H. J., and Madoff, S. J., *J. Bact.,* 1950, v59, 755.

Received September 19, 1957. P.S.E.B.M., 1957, v96.

Chlortetracycline in the Nutrition of Guinea Pigs.*† (23537)

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Numerous reports indicate that chlortetracycline (aureomycin) is harmful to the guinea pig. Roine *et al.*(1) have reviewed the literature and have explained the toxicity of orally administered aureomycin on the basis of a change in the intestinal flora. They found in treated animals an abundance of organisms that belong to the genus *Listeria,* and believed this organism to be the cause of death. Eyssen *et al.*(2) found penicillin, baci-

* Contribution from Mo. Agric. Exp. Station, Journal Series No. 1776. Approved by the Director.

† The aureomycin HCl was generously supplied through the courtesy of Dr. T. H. Jukes, Lederle Laboratories Division, Amer. Cyanamid Co., Pearl River, N. Y.

TABLE I. Chlortetracycline and Growth Rate of Weanling Male Guinea Pigs.

Chlortetracycline HCl, mg/kg diet	No. of animals	Avg daily gain, g (8 wk)
A. Purified diet		
0	12	6.3
25	8	5.5
50	4	6.5
75	8	5.3
100	4	6.4
200	4	5.7
B. Stock diet		
0	4	5.9
25	12	6.0

tracin, streptomycin and chlortetracycline to be toxic when administered singly or in various combinations, but their observations did not correspond to those of Roine *et al.*(1). They were unable to isolate *Listeria monocytogenes,* and penicillin and chloramphenicol did not protect against chlortetracycline. Chlortetracycline is not toxic under all laboratory conditions. Hewes(3) observed that it stimulated the growth of weanling guinea pigs and had no adverse effect on reproduction.

The purpose of this communication is to present additional evidence that chlortetracycline is not harmful to guinea pigs under all conditions and, in fact, is helpful in the control of certain infections. Chlortetracycline has been fed to guinea pigs in this laboratory for a period of about three years without evidence of toxicity. The experimental data presented here show its effect on growth and reproduction.

Methods. Two types of diets were used. One was a practical type diet composed of ground wheat 26; wheat germ 6; alfalfa meal 34; dried yeast 15; iodized salt 1; steamed bone meal 2; soybean oil meal 15; vitamin A-D concentrate 1; $MnSO_4 \cdot H_2O$ 0.025 and ascorbic acid 0.05%. The purified diet was similar to ration 3884 previously described (4), but contained 2.7% potassium acetate and 0.5% magnesium oxide in place of an equal amount of sucrose. For growth studies, groups of male guinea pigs weighing from 170 to 200 g were fed the respective diets for eight weeks. Feed and water were supplied *ad libitum* and the animals were weighed weekly. The reproduction data are based on all animals in the stock breeding colony during two periods—one year before the use of chlortetracycline and the year following. It was included in the stock diet at a level of 25 mg/kg. The animals were kept in battery cages with one male and three females per cage. The litters were born in the colony cage so that mating usually occurred immediately after parturition.

Results. Chlortetracycline was first used in the colony to treat an acute infectious enteritis of unknown etiology. It was effective in controlling this epidemic and it seemed desirable to include the antibiotic in the diet routinely. Since chlortetracycline had been reported to be toxic to guinea pigs, it was fed to weanling animals at different levels to check toxicity under our conditions. The results of this trial are shown in Table I. The number of animals was small and consequently there was a high variability in rate of growth, but under the conditions, chlortetracycline had no significant effect on rate of gain. There was no mortality during the eight-week period. In view of these results, 25 mg/kg of diet seemed well within the tolerance of the guinea pig and this level has been included in experimental diets fed to over 1000 weanling guinea pigs with no obvious adverse effect. In fact, it appears helpful in the control of certain types of infectious enteritis, but it has not prevented *Salmonella* infections.

The data in Table II show the reproductive

TABLE II. Effect of Chlortetracycline on Reproductive Performance of Guinea Pigs.

	Period I*	Period II†
Chlortetracycline HCl	0	25 mg/kg
No. females	161	138
No. mortalities	94	51
Cases of lymphadenitis	21	1
No. of abortions	24	7
No. of litters	352	248
No. of young born	998	822
% weaned	91	97
Avg birth wt (g):		
2 in litter	117 (75)‡	117 (48)
3 " "	97 (120)	107 (95)
4 " "	98 (61)	96 (49)

* Period I extended from 9/1/53 to 9/1/54.

† Started aureomycin supplement 9/1/54. Period II from 10/1/54 to 9/18/55.

‡ No. in parentheses indicates No. of litters.

performance of the stock colony during the year before use of chlortetracycline and the year following. The number of females indicated includes all animals that were in the colony during the year regardless of the period of time. The number in period I was greater because of higher mortality and hence a larger number of replacements. During period I, about 13% of the animals were affected with cervical lymphadenitis‡ or lumpy jaw, but there was only 1 case after the use of chlortetracycline. Since that time there have been no cases in the colony. The percentage of abortions was markedly lower in period II. The litter size, birth weight and weaning percentage were slightly, but perhaps not significantly, higher during period II. At the present time a large proportion of our experimental animals are among the fourth generation since chlortetracycline has been routinely used in the colony diet.

In view of the observations of Roine *et al.* (1), one might suspect that the successful use of dietary chlortetracycline may be due to the absence of certain types of bacteria in the intestinal flora of our animals. Listeria was thought to be the causative agent in the Finland colony. In view of widespread occurrence of *Listeria mōnocytogenes,* it seems unlikely that our colony is free of this organism. In fact, in at least one case it has been cultured from an animal during a routine pathological check.

Summary. Chlortetracycline hydrochloride has been included in various diets of a guinea pig colony for 3 years without evidence of toxicity. It had no effect on growth rate and caused no mortality when added to a purified diet at levels from 25 to 200 mg/kg. At the routine level of 25 mg/kg, the antibiotic decreased abortions and adult mortality and eliminated cervical lymphadenitis from the breeding colony. Although chlortetracycline may be toxic to guinea pigs under some conditions, in our laboratory it has been beneficial in the maintenance of a breeding colony and has been used routinely in purified experimental diets for growth studies.

‡ The causative organism, which belonged to the genus Streptococcus, was isolated and identified by Dr. L. D. Kintner, Veterinary Pathology Department.

1. Roine, P., Ettala, T., Raitio, A., and Vartiovaara, U., *Brit. J. Nutrition,* 1955, v9, 181.

2. Eyssen, H., De Somer, P., and Van Dyck, P., *Antibiotics and Chemotherapy,* 1957, v7, 55.

3. Hewes, C. G., *J. Nutrition,* 1955, v57, 353.

4. O'Dell, B. L., Vandepopuliere, J. M., Morris, E. R., and Hogan, A. G., PROC. SOC. EXP. BIOL. AND MED., 1956, v91, 220.

Received August 16, 1957. P.S.E.B.M., 1957, v96.

Accumulation of Calcium^{45} by Salivary Glands. (23538)

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Many investigators have compared Ca^{45} exchange in various tissues(1,2,3), but none appear to have included the salivary glands in their studies. Chemical determinations of calcium concentration in the salivary glands of cats has revealed that the concentration was approximately twice that of plasma(4). In dogs, no difference was apparent(4). Histochemical determination of calcium in mouse tissues did not reveal a greater concentration in salivary glands than in other tissues(5). The present report compares the changes in specific activity with time after administration of calcium^{45} in the submaxillary gland, kidney, and blood of female rats. Total calcium in submaxillary glands, kidneys, and blood of rats is also determined.

Methods. Calcium^{45} chloride* in a dose of 10 μc was given to ether-anesthetized 6-month-old virgin female Long-Evans rats by injection into a foot vein. At intervals from

* Calcium^{45} was purchased from Union Carbide Nuclear Co., Oak Ridge, Tenn. under license number 4-918-1. The specific activity was 92 Mc/g.

1 minute to 48 hours animals were etherized and organs removed, blotted, and weighed in tared 10 x 75 mm boro-silicate glass tubes. Blood was also collected and weighed in the same way. Samples were wet-ashed at temperatures below 120°C with fuming nitric acid, perchloric acid, and 30% hydrogen peroxide. Total calcium was analyzed by flame photometry using a Beckman DU, oxy-acetylene burner, and a specially built high-sensitivity, stable, photomultiplier recording attachment.† Emission interference was estimated by the addition of a known amount of calcium to the unknown and repeating the analysis(6). Exhaust gases from the flame were passed by suction through a double thickness of Fiberglas No. PF105 to remove Ca^{45}.‡ Radioactivity of the calcium present in each sample was determined by oxalate precipitation of an aliquot after addition of sufficient carrier calcium to assure complete recovery. The precipitate was spread on copper planchets and counted with an end-window GM tube. Samples were counted in the infinitely thin region (less than 1 mg/cm^2) as shown by recoveries of added Ca^{45}. Initially, samples were taken of liver, kidney, submaxillary gland, parotid gland, pancreas, ovary, and blood, but for comparison, only blood, kidney, and submaxillary gland will be discussed. Specific activities in liver, pancreas, and ovary were similar to kidney while parotid gland was similar to submaxillary gland.

Results. 1) Calcium concentration in rat organs: In 22 normal female Long-Evans rats, the mean and standard errors of stable calcium concentrations were as follows: Blood 3.66 ± .14 meq/kg; kidney 4.0 ± 0.1 meq/kg; submaxillary gland 21.6 ± 1.0 meq/kg.

2) Exchange of Ca^{45} in rat organs: Table I details the changes in specific activity in kidney, blood, and submaxillary gland at various times after intravenous administration of 10 μc $Ca^{45}Cl_2$. Differences in exchange by kidney and submaxillary glands are obvious at 5 minutes and at 8 hours. The rapid fall in blood specific activity represents exchange of calcium^{45} with bone calcium.

TABLE I. Specific Activity* in Blood, Kidney, and Submaxillary Gland at Various Times after Intravenous Administration of 10 μc Ca^{45} i.v. (3 Rats at Each Time Interval, Each Line Represents 1 Animal).

Time	Blood	Kidney	Submaxillary gland
5 min.	249	160	51
	249	175	42
	235	215	77
1 hr	45	44	29
	50	43	30
	43	67	19
4	33	32	49
	29	24	43
	35	32	50
8	17	22	130
	28	39	70
	45	48	76
12	15	12	27
	17	17	32
	13	10	32
18	41	35	39
	22	22	33
	24	39	45
24	7.5	9.5	12
	4.4	7.2	9.3
	4.5	7.7	8.8
48	2.7	2.3	4.0
	1.9	4.3	4.0
	7.2	6.2	12.0

* Counts/sec./meq Ca.

Because the specific activity level in blood of different animals was variable, the ratios of specific activity in various organs in the same animal was used as a better means of comparison. Thus, retention of Ca^{45} in one organ as compared to blood or to another organ would give a ratio greater than one. On the other hand, delayed exchange in an organ would give a ratio less than one. Table II presents the ratios of specific activity of Ca in the submaxillary gland as compared to the kidney and blood, and the ratio of specific activity in kidney as compared to blood.

The ratios of specific activity of Ca^{45} in submaxillary gland as compared to kidney or blood shows a rise from 5 minutes to 8 hours. These ratios then fall and at 18 hours the difference between blood, kidney, and the submaxillary gland is no longer significant. The probability of chance occurrence of these dif-

† Description available on request.

‡ Filter designed by Mr. Charles Lapple, Stanford Research Institute, Menlo Park, Calif. Fiberglas supplied by Fiberglas Engineering Supply Division, Owens-Corning Glass, San Francisco, Calif.

TABLE II. Ratios* of Specific Activities of Submaxillary Gland as Compared to Blood or Kidney, and of Kidney Compared to Blood at Various Times after Intravenous Injection of 10 μc Ca^{45} (6 Rats at Each Time Interval).

	Submaxillary gland		Kidney
	Kidney	Blood	Blood
1 min.			.96 ± .06
5	.20 ± .05	.18 ± .04	.90 ± .09
4 hr	1.40 ± .10	1.30 ± .20	1.00 ± .10
8	2.5 ± .7	2.9 ± 1.0	1.1 ± .1
12	2.06 ± .13	2.2 ± .25	.97 ± .06
18	1.2 ± .1	1.2 ± .2	1.3 ± .3

* Mean ± stand. error.

ferences was less than .03 at 5 minutes, 8 hours, and 12 hours. The differences at the other time intervals were not less than .05. The data indicate a significant delay in the exchange of Ca in the submaxillary gland. The equilibration time appears to be greater than 4 hours, although exact determination is difficult since the blood specific activity is falling rapidly.

In another experiment, the rapidity of exchange in the kidney was determined by killing the rats 1 minute after the injection of Ca^{45}. The mean ratio of kidney specific activity to blood specific activity in 6 rats was 0.96 with a standard error of ± .06, indicating that exchange was complete even at this short time interval.

Discussion. Various mechanisms can be postulated to explain the slow exchange of radio-calcium in the submaxillary gland. First, the slow exchange may represent a metabolically dependent, rate-limiting process for exchanging calcium across the cell membrane or the membrane of an intracellular particle. The fact that total calcium in the submaxillary gland is approximately 5 times as high as in the kidney indicates fundamental differences in the way the two tissues handle calcium.

Secondly, the slow exchange may represent a slow rate of dissociation of a tightly bound Ca complex either inside the cell or the cell surface. The specific activity ratio at 5 minutes between actively exchanging calcium in the submaxillary gland and total kidney calcium was recalculated on the assumption that the actively exchanging calcium in the gland was equal to total kidney calcium. This recalculated ratio was .80 ± .06 indicating that the exchange was still slower than kidney.

Thirdly, the slower exchange of Ca^{45} in the submaxillary gland could also be explained if the blood flow per unit weight in the gland was extremely small as compared to the kidney. Ca^{45} exchange within the kidney is complete in one minute, and presuming a renal blood flow (RBF) of 2.5 ml/g/minute this would indicate that a blood flow of 2.5 ml was sufficient to equilibrate 4.02 μeq of Ca (kidney Ca conc. $= K_c$). Since the submaxillary gland contains 21.6 μeq Ca/g (submaxillary gland Ca conc. $= S_c$), a flow of 13.5 ml of blood should be sufficient for equilibration (S_c/K_c x RBF). If an equilibration time (ET) of 4 hours is accepted, then blood flow would need to be as low as 3.3 ml/g/hour $\left(\frac{S_c/K_c \times RBF}{ET}\right)$ to be a limiting factor. Burgen(7) found the venous outflow rate from the unstimulated submaxillary gland in dogs was 13 ml/g/hour. This presumably is low since it was taken after surgical isolation of the blood vessels. Thus, if the submaxillary circulatory volume of rats is at all comparable to that in dogs, the rate of circulation is apparently not the only factor controlling the slow exchange of Ca^{45} in the submaxillary gland.

Studies are being made to elucidate the mechanisms controlling the high salivary gland Ca, the slow exchange with blood Ca, and of the part this Ca plays in the secretion of saliva calcium.

Conclusion. 1. Ca^{45} accumulation and loss in the submaxillary gland is much slower than in the kidney. Equilibrium between the gland and blood is reached approximately 4 hours after administration, while at 8 hours the specific activity of calcium in blood and kidneys has fallen to less than one-half that in the submaxillary gland. Equilibrium is again reached at 18 hours. 2. In female Long-Evans rats, total calcium in the submaxillary gland was 21.6 ± 1.0 meq/kg, in the kidney 4.0 ± 0.1 meq/kg and in blood 3.66 ± .14 meq/kg.

1. Campbell, W. W., and Greenburg, D. M., *Proc. Nat. Acad. Sci. U. S.,* 1940, v26, 180.

2. Norris, W. P., *Conference on Biological Applications of Nuclear Physics,* Brookhaven National Lab., 1948, 78.

3. Imanaga, H., Cho, M., Kurita, I., Nozaki, N., *Nagoya J. Med. Sci.,* 1952, v15, 141.

4. Wills, J. H., *Am. J. Physiol.,* 1941, v135, 164.

5. Rohdenburg, G. L., Geiger, J., *Arch. Path. Lab. Med.,* 1928, v6, 215.

6. Hubener, H. J., *Klin. Wochschr.,* 1953, v31, 1095.

7. Burgen, A., *J. Physiol.,* 1956, v132, 20.

Received September 16, 1957. P.S.E.B.M., 1957, v96.

Host Resistance to Hemorrhagic Shock X. Induction of Resistance by Shock Plasma and by Endotoxins.* (23539)

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The blood and tissues of an animal (dog, rabbit) in prolonged hemorrhagic shock contains a toxin which is the product of bacterial activity(1). It accumulates in consequence of injury to the antibacterial defense mechanisms and produces irreversibility to transfusion and death. If the integrity of the antibacterial mechanisms could be sustained, so that these toxins would be neutralized, or their production suppressed, the shock, even if severe and prolonged, should remain reversible to transfusion.

Accordingly, experiments were designed to explore the possibility of inducing tolerance to hemorrhagic shock, *i.e.* preventing development of irreversibility to transfusion, by prior exposure to known bacterial endotoxins. Should tolerance to shock be so induced, the same result might also be obtained by prior exposure to plasma from irreversibly shocked animals, if the toxic factor in this plasma which produces irreversibility is in fact a bacterial endotoxin. The data in this communication demonstrate that protection against irreversibility to transfusion and death from severe and prolonged hemorrhagic shock can be secured by prior treatment with plasma from severely shocked animals, as well as by prior treatment with known endotoxins.

Method. Rabbits were selected as the most suitable species in which to produce tolerance to endotoxin, and subsequently to gauge the effect of such tolerance on the course of hemorrhagic shock. The MLD/100 of Difco *E. coli* Endotoxin (Batch No. 0127-B8) was found to be 2 mg/kg body weight in normal rabbits. The MSD/100 (maximal surviving dose, *i.e.* the highest dose that can be given with a zero mortality rate) was 0.03 mg/kg. Seven groups of adult albino rabbits (average weight 2-3 kg) were trained for 3 days so that spontaneous fever would not occur in response to the required manipulations(2). Each rabbit then received a daily dose (1 MSD/100 or less—see Table I) of endotoxin intravenously for 7 successive days. In every instance observations were made on the fever response, and on the total and differential leucocyte count during the subsequent 6 hours. The fever response was characteristic for endotoxin and showed the anticipated daily decline, so that by the seventh day the curve was characteristic of induced tolerance to the endotoxin (Fig. 1). On the eighth day hemorrhagic shock was produced by a standard method described elsewhere(3), and tolerance to shock observed, especially with regard to rate of "take-up" (*i.e.* spontaneous return of the shed blood to the animal from the elevated reservoir), pressor response to transfusion, survival time and survival rate. One of the 7

* Aided by grant from National Heart Inst., N.I.H., Bethesda, Md., and by Contract with Office of Surgeon General, U. S. Army.

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TABLE I. Survival of Rabbits Subjected to 6 Hours of Hemorrhagic Shock following Pretreatment with 7 Daily Injections of Endotoxin* or Shock Plasma.

Exp.	Daily dose, mg/kg	Total No. Animals	Survivors
1	.03 endotoxin	6	6
2	.015 "	6	5
3	.0075 "	6	5
4†	.002 "	7	6†
5	.001 "	6	5
6	.0005 "	10	3
7	13 ml shock plasma	10	8

* MLD = 2 mg/kg/b.w.

† 3 of these 6 animals recovered from shock but died of sepsis after 48 hr. Gastrointestinal hemorrhage, characteristic of shock, was not found.

groups of rabbits was exsanguinated 30 minutes after the transfusion, and the blood so removed was tested, as described elsewhere (1),‡ for the presence of the toxin causing irreversibility. The foregoing experiments were repeated in another series of rabbits, except that, in place of endotoxin, a daily intravenous injection of 13 ml§ of irreversible shock plasma, known to contain the lethal toxin, was given for 7 successive days. If the toxin in this plasma is an endotoxin, one should expect the plasma to act like known endotoxin in respect of the induction of tolerance. Accordingly, the fever and leucocyte responses were obtained following each daily injection. Some of these rabbits were then tested for tolerance to prolonged shock. Others were put into shock for 1½ hours and compared with unprepared (*i.e.* normal) rabbits in shock for 1½ hours for their susceptibility to the toxin in shock plasma causing irreversibility.‡

Results. I. *Fever Response.* The upper 2 curves in Fig. 1 depict the characteristic course of the fever following intravenous injection of one MSD/100 or less of endotoxin on the first and on the seventh days. The lower 2 curves in Fig. 1 show the corresponding fever curves in response to shock plasma. Except for the occasional absence of the typical notch in the first day curve at about 1½ hours, they are virtually the same.

Comment. Normal plasma or plasma from normal blood standing in a reservoir for 6 hours at room temperature (to simulate the conditions of the shock experiment, in which

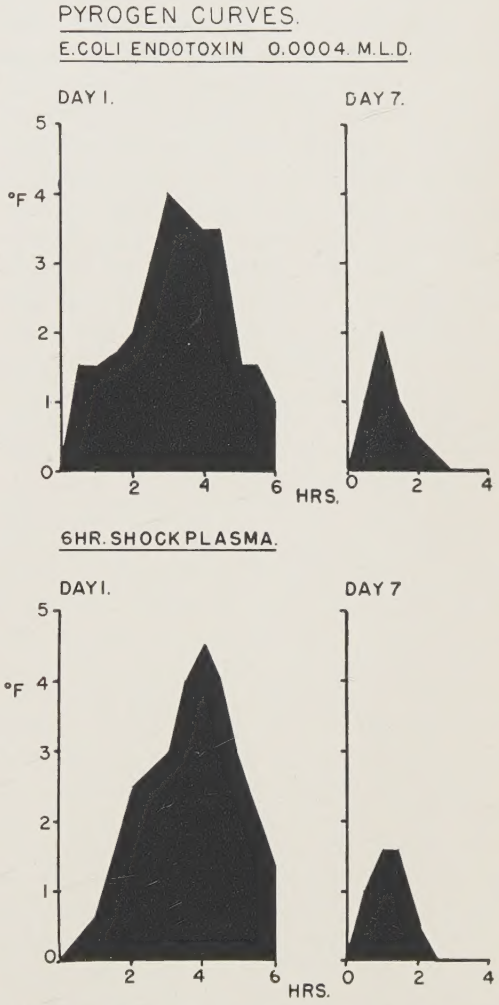

FIG. 1. Upper left curve depicts characteristic fever response of normal rabbit during 6 hr following first intrav. inj. of a sublethal dose of endotoxin. Upper right curve shows fever response on the seventh of seven successive daily inj. of the same dose of endotoxin. Lower curves depict corresponding fever response curves to 13 ml irreversible shock plasma given in place of the endotoxin.

‡ A Normal rabbit transfused after 1½ hours of hemorrhagic shock with its own blood or plasma recovers promptly and survives without damage to the gut. If transfused with toxic blood or plasma, instead of its own shed blood, the response to transfusion is poor, the gut is hemorrhagic and death follows.

§ Previous experience had shown that as little as 13 ml of such plasma could kill a 1½ hour shock recipient.

shed blood, which is later returned to the animal, might become contaminated with pyrogens in the reservoir) were uniformly non-pyrogenic (six experiments).

II. *Leucocyte Response* (Total and Differential Counts). A decline in the total and granulocyte count begins almost immediately following injection of endotoxin. The granulocytopenia reaches its lowest level within 60 to 120 minutes. Recovery of the normal total and differential counts begins after this time and is back to normal after 3 or more hours, depending on dose of endotoxin given. The injection of shock plasma produces a similar response. The acquisition of tolerance to endotoxin is reflected in the declining fever response to repeated daily injections of endotoxin or shock plasma. It is not reflected in the granulocytopenic response, which is quantitatively the same from day to day. Hemorrhagic shock also produces a granulocytopenic response in both tolerant and non-tolerant rabbits. But the drop in the count is less in the tolerant animal, and there is a rapid return to a normal or above normal count during the shock period (Fig. 2B). In the non-tolerant animal the count remains at its lowest level until death (Fig. 2A).

III. *Course of Hemorrhagic Shock.* The maximum bleeding volume, *i.e.* maximum amount of blood lost into the reservoir (some 60-80 ml), is the same in rabbits tolerant to endotoxin or shock plasma as in normal rabbits. As described elsewhere(3), little or none of the shed blood in the elevated reservoir connected to the femoral artery returns spontaneously during the first 1½-2 hours of shock in the normal, *i.e.* the non-tolerant animal. The same is true for the tolerant animal. But if the shock is allowed to continue, the course of events differs markedly between these 2 preparations. In the non-tolerant animal, blood returns spontaneously at a variable rate, and when 40-50% of the total has returned, forced infusion of the remainder is followed by a transient pressor response and death. Spontaneous return of 40-50% of the blood usually occurs within 4-5 hours. Even if less than this volume has returned by this time, forced infusion of the remainder is not curative. The spontaneous return of blood ("take-up") is evidence of failing vasoconstrictor compensation. As a rule, the more rapid the take-up, the shorter the survival after normovolemia has been restored. In most instances death occurs within 12 hours. Only a few survive up to 24 hours. None survive beyond the sixth hour if none of the shed blood is force-infused. In the rabbit which is tolerant to endotoxin or to shock plasma no blood from the reservoir returns to the animal during the period of exposure to shock (6 hours). Transfusion after 6 hours of the entire content of the reservoir produces an immediate and sustained pressor response, with prompt and full recovery in 70% of rabbits tolerant to endotoxin, and 80% of

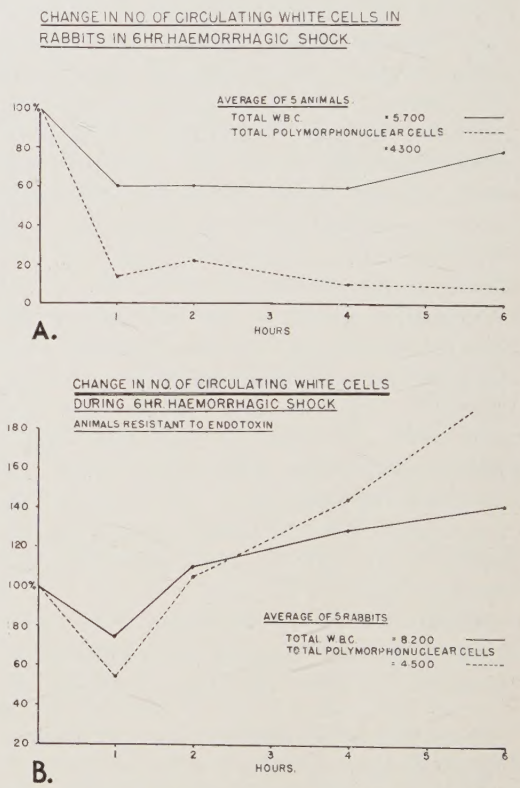

FIG. 2. A. Dotted line shows that sharp drop in granulocyte count of normal rabbits during prolonged hemorrhagic shock persists for duration of the shock. Return of the total white count to normal by the sixth hr is due to an absolute lymphocytosis. B. Rabbit resistant to endotoxin shows a much milder and very transient leucopenia, and a capacity while still in shock to respond with a granulocytosis.

rabbits tolerant to shock plasma (Table I). The non-survivors live for an average of 24 hours.

In Fig. 3 the solid line curve is typical of the rate of take-up in a normal rabbit in hemorrhagic shock at 50 mm Hg. The slope of the curve varies somewhat from one group of experiments to another, and in the warmer seasons the slope is steeper. The broken line is the curve for the rabbit resistant to endotoxin or shock plasma. Its horizontal shape signifies sustained compensation, *i.e.* no take-up for the full 6 hours.

Comment. The dose of endotoxin used to produce tolerance varied from 1/75-1/4,800 of an MLD/100. The tolerance to shock, as judged by mortality rate, was about the same for all doses down to and including 1/2400 of an MLD/100.‖ The shock plasma was as efficient as endotoxin in producing tolerance to shock. But it is difficult to say whether one can justifiably assay the endotoxic potency of the shock plasma in terms of the quantitative data on the effectiveness of endotoxin.

IV. *Toxicity of Blood of Rabbits Resistant to Shock.* The blood taken after 6 hours in shock from one group of endotoxin-tolerant rabbits, and tested for the presence of the toxin causing irreversibility, was non-toxic in seven of nine experiments.

V. *Susceptibility of Tolerant Rabbits Transfused After 1½ Hours in Shock to Toxic Plasma.* In this experiment 5 rabbits tolerant to shock plasma and, therefore, tolerant to shock, were put into shock for 1½ hours, then transfused with plasma known to be toxic to non-tolerant rabbits. Four out of the 5 survived, whereas only one of 12 non-tolerant animals survived similar treatment (1).

Comment. From the first of the latter 2 observations one may infer that the animal tolerant to shock is tolerant by virtue of its ability either to prevent production of toxin or to neutralize toxin if produced. That at least the latter is true is demonstrated by the second of these 2 observations.

Discussion. The evidence that a bacterial factor is involved in the death from hemorrhagic shock(4,5,6) is strongly supported by the foregoing observation that induction of tolerance to endotoxins induces tolerance to hemorrhagic shock. This observation also explains development of tolerance to hemorrhagic shock as well as drum shock in rats as a result of prior exposure to a sublethal amount of drum trauma(7), since there is reason to believe that bacterial invasion is facilitated by such trauma(8).

The fact that shock plasma contains a substance which acts like endotoxin in respect of the fever response to intravenous injection, and in respect of the capacity to induce tolerance to hemorrhagic shock, is strong evidence that this substance is an endotoxin. Shear and his co-workers, however, suggest that certain tissue polysaccharides which might be released during shock as a result of tissue anoxia, might be the agents responsible for irreversibility to transfusion.** For they have isolated polysaccharides from many tissues and from plants, which, like the levans prepared by Hestrin(9) and like endotoxin, bind Properdin in the presence of complement and magnesium(10), and otherwise behave like endotoxins(11).¶ Elsewhere we have stated why such substances, if present, do not account for irreversibility(1). In particular, we find it difficult to accept this hypothesis because non-absorbable antibiotics, given orally in advance of shock, which cannot prevent severe and prolonged shock from releasing such substances, do prevent irreversibility. Most of the bloods from rabbits and dogs pretreated with such antibiotics and shocked thereafter, are as harmless to recipient test animals as is normal plasma(1). Hence the lethal substance is exogenous, and the only lethal substance that non-absorbable anti-

‖ In Exp. IV (Table I) the 3 rabbits which died at 48 hours behaved during shock and in response to transfusion as well as the others. Intestinal mucosa at death was of normal appearance. Their death was attributable to severe sepsis in the groin wounds.

** Personal communication.

¶ Evidence that the toxic properties of these compounds are not in fact due to contaminating endotoxins has not yet been provided.

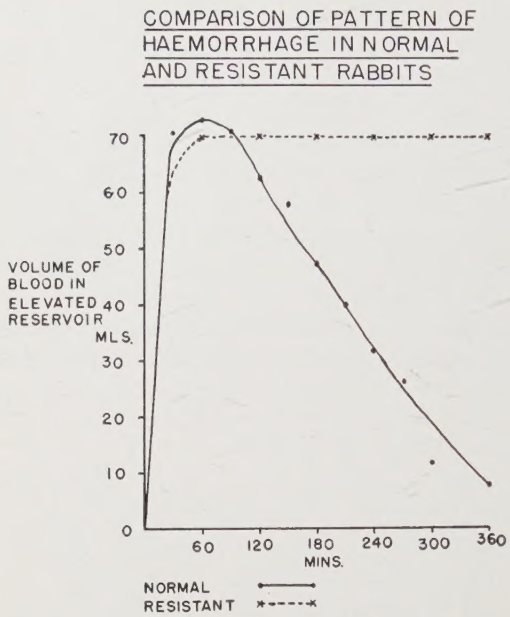

FIG. 3. Rise in the curves represents rate of bleed-out from the femoral artery of the rabbit into a reservoir elevated so as to keep the blood pressure constant at 50 mm Hg. Difference in the 2 curves thereafter represents difference in the rate at which blood returns spontaneously from reservoir to circulation.

biotics put into the gut are likely to eliminate is bacterial endotoxin.

Since shock plasma contains a transferable pyrogen which is nullified by tolerance, the pyrogen, like the toxin, is exogenous(12). Non-absorbable antibiotics eliminate the pyrogen (unpublished data) as well as the toxin from shock plasma. Therefore, it can be assumed that the pyrogen, like the toxin, is of bacterial origin, and is presumably the toxin itself.

Summary and conclusions. Rabbits made tolerant to bacterial endotoxin tolerate an otherwise lethal exposure to hemorrhagic shock. Whereas normal, *i.e.* non-tolerant, rabbits are dead after 6 hours of shock in spite of having received all the blood removed, tolerant rabbits are in satisfactory condition after 6 hours in spite of having received none of the blood removed. They respond promptly when transfused at the end of 6 hours and recover completely. The same results are secured in rabbits made tolerant to, *i.e.* pretreated with, shock plasma. Therefore, there is reason to consider the toxin in shock plasma an endotoxin. In the rabbit tolerant to endotoxin the blood taken after 6 hours in shock is free of the toxin causing irreversibility, which is in the blood of non-tolerant rabbits in shock. In the rabbit tolerant to shock the granulocytopenic response is distinctly different from that in the non-tolerant animal. The response is less intense and recovery to a normal or above normal count occurs while the rabbit is still in shock. In the non-tolerant rabbit the granulocytopenia persists throughout the shock period. The induction of tolerance to hemorrhagic shock by shock plasma as well as by endotoxin supports the view that a bacterial factor is responsible for irreversibility to transfusion in hemorrhagic shock. The fact that rabbits tolerant to hemorrhagic shock can resist a dose of shock blood or plasma that is lethal to non-tolerant rabbits, and that their bloods do not contain the toxin which develops in non-tolerant rabbits, supports the view that tolerance to hemorrhagic shock is due to the maintenance or enhancement of the normal capacity to neutralize bacterial endotoxin.

1. Schweinburg, F. B., Shapiro, P. B., Frank, E. D., and Fine, J., PROC. SOC. EXP. BIOL. AND MED., 1957, v95, 646.

2. Grant, F., *Am. J. Physiol.,* 1950, v160, 285.

3. Fine, J., *Ann. N. Y. Acad. Sci.,* 1952, v55, 429.

4. ———, *ibid.,* 1956, v66, 329.

5. Jacob, S., Weizel, H., Gordon, E., Korman, H., Schweinburg, F. B., Frank, H. A., and Fine, J., *Am. J. Physiol.,* 1954, v179, 523.

6. Frank, E. D., Kaufman, D., Korman, H., Schweinburg, F. B., Frank, H. A., and Fine, J., *ibid.,* 1954, v179, 532.

7. Shorr, E., Zweifach, B., *Shock and Circulatory Homeostasis,* Josiah Macy Jr. Fn., 1952, P100.

8. Friedman, E. W., Schweinburg, F. B., Yashar, J., and Fine, J., *Am. J. Physiol.,* 1957, v189, 197.

9. Hestrin, S., *N. Y. Acad. Sci.,* 1956, v66, (2) 401.

10. Pillemer, L., *ibid.,* 1956, v66, (2) 233.

11. Landy, N., and Shear, M. J., *J. Exp. Med.,* 1957, v106, 77.

12. Bennett, I. L., Jr., and Beeson, P. B., *J. Exp. Med.,* 1953, v98, 493; Atkins, E., and Wood, W. B., Jr., *J. Exp. Med.,* 1955, v102, 499.

Received July 29, 1957. P.S.E.B.M., 1957, v96.